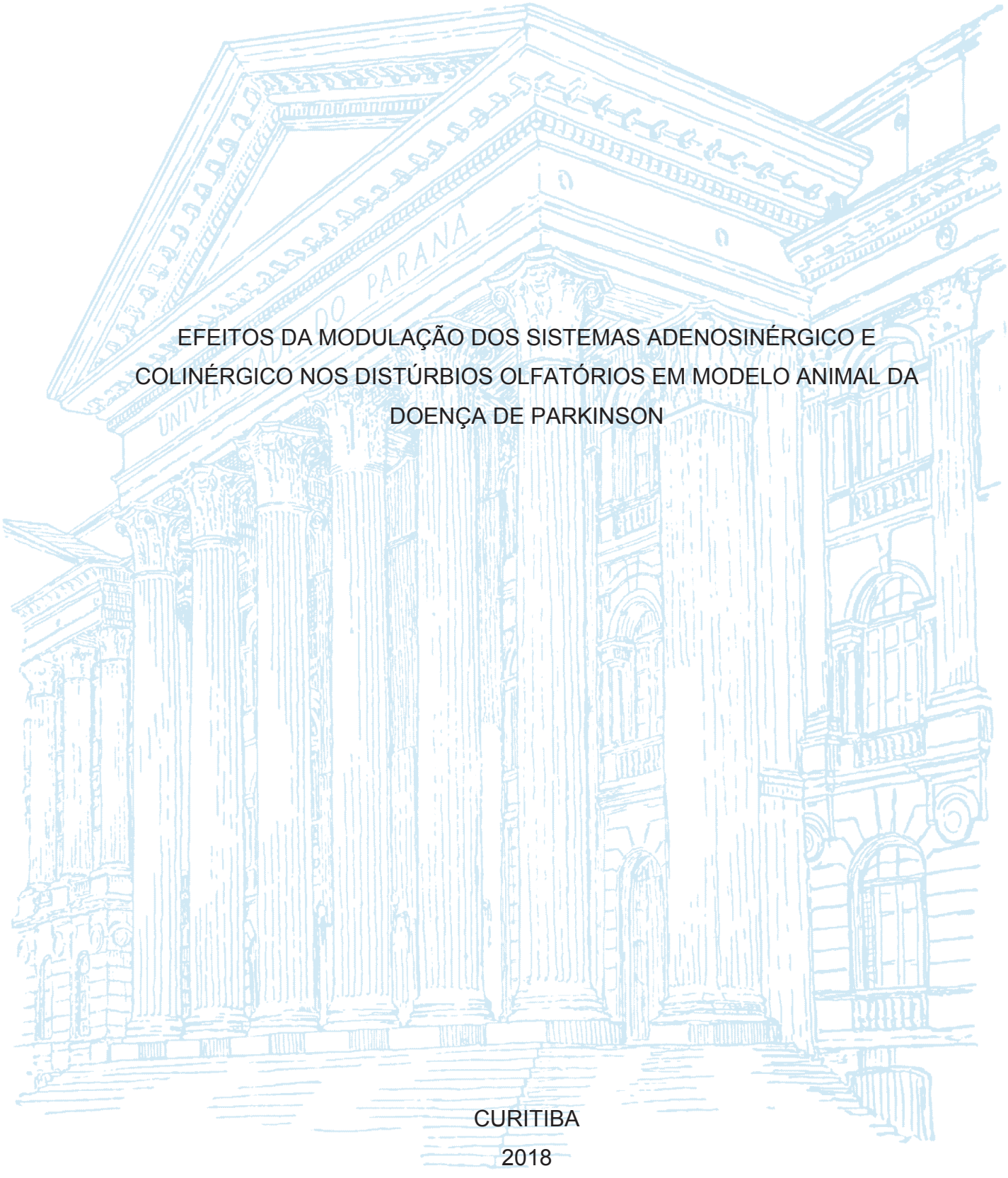
UNIVERSIDADE FEDERAL DO PARANÁ

LAIS SOARES RODRIGUES



EFEITOS DA MODULAÇÃO DOS SISTEMAS ADENOSINÉRGICO E COLINÉRGICO NOS DISTÚRBIOS OLFATÓRIOS EM MODELO ANIMAL DA DOENÇA DE PARKINSON

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Farmacologia.

Orientador: Prof. Dr. Marcelo M. S. Lima

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LAIS SOARES RODRIGUES** intitulada: **Efeitos da modulação dos sistemas adenosinérgico e colinérgico nos distúrbios olfatórios em modelo animal da doença de Parkinson**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 28 de Setembro de 2018.

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RESUMO

Os distúrbios olfatórios presentes na doença de Parkinson (DP) acometem cerca de 90% dos pacientes, atingindo mais homens do que mulheres. Esses distúrbios são considerados prodrômicos, pois se iniciam antes da fase motora clássica da doença. Apesar dos distúrbios motores serem considerados os mais incapacitantes para os pacientes, os distúrbios olfatórios prejudicam a qualidade de vida e possibilitam um diagnóstico precoce, uma vez que a doença ainda hoje é diagnosticada em fase avançada, na maioria das vezes. A terapia clássica utilizada por pacientes Parkinsonianos não tem efeito sobre os distúrbios olfatórios e, além disso, não é utilizado nenhum tratamento específico para esse déficit não motor. Estudos epidemiológicos e pré clínicos sugerem potencial efeito neuroprotetor de substâncias como cafeína e nicotina, porém, sem demonstrações de seus efeitos no prejuízo olfatório relacionado à DP. Dessa forma, buscamos investigar os efeitos da modulação dos sistemas adenosinérgico e colinérgico diante da disfunção olfatória causada pelo modelo animal de DP induzido por rotenona intranigral. Ratos machos e fêmeas foram submetidos a cirurgia estereotáxica para lesão da substância negra pars compacta (SNpc) e, em seguida, foi realizado um tratamento prolongado de 7 dias com cafeína e/ou nicotina ou agudo com agonista e antagonista A2a no bulbo olfatório (BO). Todos os animais foram submetidos à testes comportamentais e seus BOs foram dissecados ou perfundidos para posteriores análises de western blot ou imunohistoquímica. Os resultados demonstraram que tanto a cafeína quando o antagonista A2a seletivo reverteram o prejuízo olfatório provocado pela lesão, em machos. Por outro lado, tanto a nicotina quanto o agonista A2a prejudicaram a discriminação olfatória, também em machos, com aumento da fosforilação de tirosina hidroxilase (TH) exacerbada, para a nicotina. Aparentemente, o modelo utilizado não afetou significativamente a função olfatória de fêmeas, tampouco a memória de reconhecimento social em ambos os sexos. Tanto a cafeína quanto a nicotina foram capazes de restaurar parcialmente e totalmente a degeneração na SNpc, respectivamente, indicando um potencial neuroprotetor neste modelo. Concluindo, os resultados sugerem que existem diferenças entre os sexos no modelo animal utilizado, semelhante ao que ocorre em uma população humana, sendo necessárias terapias diferenciadas por sexo. Ainda, sugerimos, indiretamente, que o sistema adenosinérgico está intimamente envolvido no processo olfatório, possivelmente interagindo com o sistema dopaminérgico.

Palavras-chave: Doença de Parkinson. Distúrbio olfatório. Rotenona. Cafeína. Nicotina.

ABSTRACT

Olfactory disorders present in Parkinson's disease (PD) affect about 90% of patients, reaching more men than women. These disorders are considered prodromal, since they begin before the classic motor phase. Although the motor disturbances are considered the most disabling disorders for patients, the olfactory impairment affects the quality of life and is important for early diagnosis, since the disease is still diagnosed at an advanced stage, most of the time. The classical therapy used by Parkinsonian patients has no effect on olfactory disorders and, in addition, no specific treatment is used for this non-motor deficit. Epidemiological and preclinical studies suggest a potential neuroprotective effect of substances such as caffeine and nicotine, but without demonstrated effects on the PD olfactory impairment. Therefore, we investigated the effects of modulation of the adenosinergic and cholinergic systems in the face of the olfactory dysfunction caused by intranigral rotenone. Male rats and females were submitted to a stereotaxic surgery for lesions of the substantia nigra pars compacta (SNpc) and then were administered prolonged treatment of 7 days with caffeine and/or nicotine or acute with agonist and A2a antagonist in the olfactory bulb (OB). All animals were submitted to behavioral tests and their OBs were dissected or perfused for subsequent western blot analysis or immunohistochemistry. The results demonstrated that both caffeine and the selective A2a antagonist reversed the impaired olfactory function in males. Otherwise, both nicotine and A2a agonist impaired olfactory discrimination, also in males, with increased tyrosine hydroxylase (HT) phosphorylation for nicotine. Apparently, the model used does not significantly affect the female olfactory function, neither the social recognition memory in both sexes. Both nicotine and caffeine were able to restore fully and partially degeneration in SNpc, respectively, indicating a potential neuroprotective effect in this model. In conclusion, the results indicate that there are differences between the sexes in the animal model used, similar to what occurs in a human population, requiring differentiated therapies by sex. Furthermore, we suggest, indirectly, that the adenosinergic system is closely involved in the olfactory process, possibly interacting with the dopaminergic system.

Key-words: Parkinson’s disease. Olfactory disturbance. Rotenone. Caffeine. Nicotine.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS – LÍNGUA PORTUGUESA

6-OHDA – 6-hidroxidopamina
ATP – Adenosina trifosfato
BO – Bulbo olfatório
CG – Camada glomerular
DA - Dopamina
DAT- Transportador de dopamina
DP – Doença de Parkinson
ERO – Espécies reativas de oxigênio
fosfo-TH – Tirosina hidroxilase fosforilada
GABA – Ácido gamma-aminobutirico
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
i.p. - intraperitoneal
MAO B – Monoaminaoxidase B
MPTP - 1-metil-4-fenil-1,2,3,6-tetrahidropiridina
m/t – mitrais/tufosas
nAChRs – Receptores colinérgicos nicotínicos
NADPH - Fosfato de dinucleótido de nicotinamida e adenina
SNpc – Substância negra pars compacta
TDO – Tarefa de discriminação olfatória
TH – Tirosina hidroxilase
TH-ir – Tirosina hidroxilase-imunorreativo
VMAT2 – Transportador vesicular de monoaminas 2

LISTA DE ABREVIATURAS – LÍNGUA INGLESA

6-OHDA – 6-hydroxidopamine
DA - Dopamine
DAT- Dopamine transporter
DMSO - dimetylsulfoxide
DP – Parkinson's disease
GABA – Gamma-aminobutyric acid
GL – Glomerular layer
i.p. - intraperitoneal
MAO B – Monoamine oxidase B
MPTP - 1-metil-4-fenil-1,2,3,6-tetrahidropiridine
nAChRs – Nicotinic acetylcholine receptors
Nicotine b.s. – Nicotine bi-tartrate salt
OB – Olfactory bulb
ODI – Olfactory discrimination index
ODT – Olfactory discrimination task
OFT – Open field test
p-TH – Tyrosine hydroxylase phosphorylated form ROS – Reactive oxygen species
SNpc – Substantia nigra pars compacta
SRT – Social recognition test
TH – Tyrosine hydroxylase
TH-ir – Tyrosine hydroxylase immunorreactive

SUMÁRIO

1 INTRODUÇÃO

1.1 DOENÇA DE PARKINSON

A doença de Parkinson (DP) é uma doença neurodegenerativa progressiva que afeta de 1 a 3% da população acima de 60 anos, sendo considerada a segunda mais prevalente no mundo, atrás apenas da doença de Alzheimer (NELSON, 1999; BLESA et al., 2012; FERESHTEHNEJAD; LÖKK, 2014; TRISTÃO et al., 2014). Essa doença foi originalmente descrita por James Parkinson em 1817, que a denominou paralisia agitante (“shaking palsy”), relatando em alguns pacientes a ocorrência de sinais como tremor em repouso, fraqueza muscular, anormalidades posturais e de marcha (DAUER; PRZEDBORSKI, 2003). A maioria dos casos de DP é de causa idiopática (LANG; LOZANO, 1998), sendo que a taxa de prevalência quanto ao sexo varia de 1,4 a 3,7 vezes, sendo a maior delas encontrada em homens do que mulheres (GILLIES et al., 2014). Essa diferença tem sido atribuída aos hormônios sexuais, como o 17 β-estradiol, o principal estrogênio presente em fêmeas de mamíferos não-gestantes, e com potencial neuroprotetor já descrito em doenças como a DP (DLUZEN, 2000; BOURQUE; DLUZEN; DI PAOLO, 2009). Dessa forma, reforça-se a importância dos estudos que envolvem a investigação de aspectos associados aos sexos, permitindo assim a busca de terapias diferenciadas e até mesmo em relação aos mecanismos envolvidos. Nesse sentido, o NIH (National Institutes of Health) incentiva pesquisas clínicas e pré-clínicas que abordem doenças, estratégias de prevenção e tratamentos sejam realizadas em ambos os sexos, avaliando possíveis dimorfismos sexuais nsses contextos.

O fenótipo motor da DP se dá, principalmente, em função da perda de neurônios dopaminérgicos presentes na substância negra pars compacta (SNpc), a qual envia projeções para o corpo estriado e outros núcleos da base sob condições normais (BARRAUD et al., 2009). Os sinais e sintomas motores característicos da DP surgem quando ocorre a morte de mais de 50% dos neurônios dopaminérgicos do mesencéfalo e perda de 80 a 90% do conteúdo de dopamina (DA) estriatal (LANG; LOZANO, 1998; LANE; DUNNETT, 2008). As manifestações motoras caracterizam-se como as mais incapacitantes para o paciente dentro do conjunto sintomatológico apresentado, sendo elas: bradicinesia, rigidez muscular, acinesia, tremor em repouso e anormalidades posturais e de marcha (AMINOFF;

FRANCISCO, 1994; FAHN, 2003). Todavia, essa doença também apresenta distúrbios não motores, como distúrbios olfatórios, do sono, da fala, autonômicos, do humor, dificuldade de mastigação, fadiga e perda de peso (POEWE, 2008; GRINBERG et al., 2010). Essas manifestações não motoras aparecem muito antes do comprometimento motor e, dessa forma, apresentam grande relevância no estudo da DP, uma vez que podem possibilitar um diagnóstico precoce dessa doença (BARRAUD et al., 2009; HAEHNER; HUMMEL; REICHMANN, 2011).

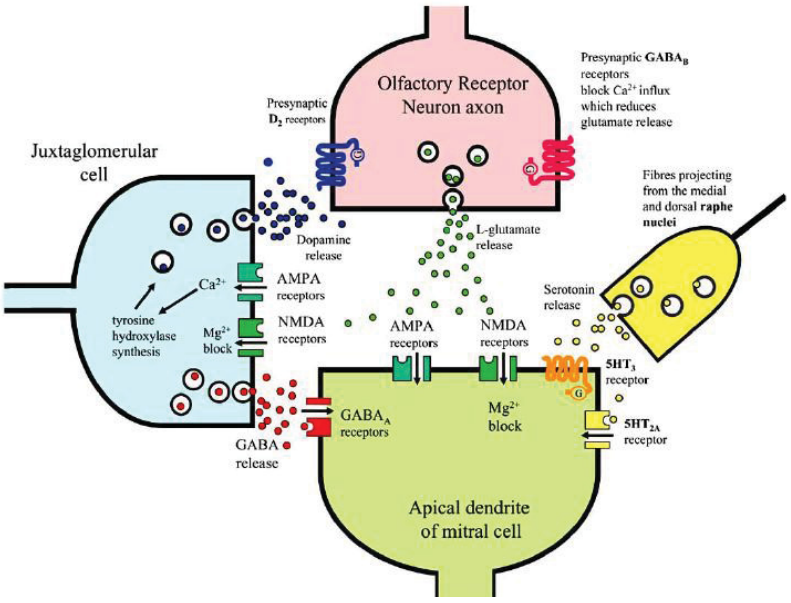
1.2 DISTÚRBIOS OLFATÓRIOS NA DOENÇA DE PARKINSON

Perdas da capacidade discriminativa olfatória ocorrem em 90% dos pacientes com DP (DOTY; DEEMS; STELLER, 1988), sendo mais pronunciadas em homens do que em mulheres (LIU et al., 2015). Os distúrbios olfatórios na DP são relatados anos ou décadas antes dos pacientes apresentarem alguma alteração motora (G. Webster Ross et al., 2008), como corroborado pela proposta de evolução topográfica da doença, de acordo com a teoria de Braak e colaboradores, que identificou uma evolução no padrão de lesões em diferentes áreas encefálicas envolvendo diversos núcleos (BRAAK et al., 2003b), coincidindo com o aparecimento de corpos de Lewy no tronco encefálico e córtex (HALLIDAY; LEES; STERN, 2011; DOTY, 2012a). Dessa forma, esses autores descreveram seis fases de evolução da doença, que se iniciaria (fases 1 e 2) em áreas associadas à olfação como o bulbo olfatório (BO) e áreas como o núcleo pedúndulo-pontino (envolvido no controle do ciclo vigília-sono) para então atingir áreas como a SNpc (fase 3-4) e córtex (fase 6) (BRAAK et al., 2003b, 2004). Recentemente, demonstrou-se a existência de uma nova via nigro-olfatória, conectando diretamente a SNpc ao BO, influenciando o desempenho olfativo no modelo animal de DP induzido por 6-hidroxidopamina (6-OHDA) intranigral (HÖGLINGER et al., 2015). Esse estudo indica que essas duas estruturas estão intimamente conectadas e regulando o processamento olfatório, sendo, portanto, intensamente afetadas durante à evolução neurodegenerativa observada na DP.

O BO é a principal estrutura do sistema olfatório, pois é nele que ocorrem as sinapses glomerulares, formadas entre os neurônios receptores de odor e células mitrais e tufosas (m/t), que são as células responsáveis por encaminhar a resposta olfatória até as áreas corticais, para a percepção do odor específico (DUDA, 2010).

O BO de mamíferos contém uma abundante população de interneurônios que expressam tirosina hidroxilase (TH), que é a enzima responsável pela síntese de catecolaminas (HOKFELT et al., 1976, 1977; HALÁSZ et al., 1981). Estes interneurônios dopaminérgicos estão presentes em grande densidade na camada glomerular (CG) do BO, bem como na camada plexiforme externa, comparativamente em menor densidade (LIBERIA et al., 2012). Na CG, a liberação de glutamato por um neurônio receptor de odor leva à despolarização de interneurônios dopaminérgicos e de células m/t. Como resposta, ocorre a liberação de DA e de ácido gamma-aminobutírico (GABA) pelos interneurônios, ativando receptores D2 nos neurônios receptores de odor e receptores GABAérgicos nas células m/t, respectivamente. Concomitantemente, ocorre uma retroalimentação negativa da atividade dopaminérgia através da ativação de receptores dopaminérgicos do tipo D2, que impedem a liberação de glutamato pelos neurônios receptores de odor, uma vez que esses receptores são inibitórios. Ainda, a ativação de receptores GABAérgicos inibitórios nas células m/t impede que a informação olfatória seja processada em áreas corticais, ocasionando a hiposmia (Figura 1) (O'CONNOR; JACOB, 2008; DOTY, 2012b).

FIGURA 1 – SINAPSES GLOMERULARES NO BULBO OLFATÓRIO



FONTE: O'Connor e Jacob (2008).

LEGENDA: Sinapse glomerular e o processamento olfatório. Os axônios dos neurônios receptores olfatórios fazem sinapses com os dendritos das células mitrais e tufosas. O L-glutamato é o neurotransmissor excitatório principal nesta sinapse, que se liga a receptores NMDA e AMPA na membrana pós-sináptica. Células justaglomerulares ou também denominadas periglomerulares são

os interneurônios inibitórios GABAérgicos/dopaminérgicos. Fibras centrífugas dos núcleos da rafe se projetam para os glomérulos modulando a atividade das células mitrais através de receptores pós-sinápticos de 5HT. Retirado de O'Connor e Jacob, 2008.

Ao contrário do que acontece na SNpc, estrutura conhecidamente atingida pela degeneração na DP, Huisman e colaboradores (2004) observaram um aumento no número de neurônios dopaminérgicos no BO de pessoas com a DP (HUISMAN; UYLINGS; HOOGLAND, 2004). Descreveu-se que a neurogênese dopaminérgica na CG do BO havia triplicado após a degeneração nigroestriatal em humanos, e que o número total de neurônios TH- imunorreativos (TH-ir) havia duplicado em comparação aos controles. Portanto, esses achados em encéfalos post mortem levaram Huisman e colaboradores a sugerir que o aumento dessas células seria uma resposta do tipo compensatória à perda de neurônios dopaminérgicos que ocorria nos núcleos da base, com este aumento levando então à perda da capacidade olfativa que coincide com os relatos de hiposmia da maioria dos pacientes Parkinsonianos (HUISMAN; UYLINGS; HOOGLAND, 2004). Além disso, propôs-se que esse aparente fenômeno de neurogênese produziria um efeito inibitório mediado pela DA, via receptor D2, na transmissão entre os neurônios receptores de odor e as células m/t nos glomérulos olfatórios (DOTY; RISSER, 1989; KOSTER et al., 1999; GUTIÈRREZ-MECINAS et al., 2005). Foi demonstrado, por outro lado, que o bloqueio de receptores D2 na CG por raclopride (antagonista dopaminérgico) poderia desencadear um aumento da liberação de glutamato em neurônios receptores de odor, levando assim à ativação de células periglomerulares e ao aumento no nível de DA e GABA liberado por estas células, promovendo, também, o prejuízo olfatório (RODRIGUES et al., 2014). Em conjunto, esses dados sugerem que é necessário um equilíbrio fino no sistema dopaminérgico para que o processo olfatório ocorra normalmente.

1.3 MODELOS ANIMAIS DE DP INDUZIDOS POR NEUROTOXINAS

A proposta de utilização de neurotoxinas para mimetizar a DP em modelos animais é importante para a pesquisa dos distúrbios apresentados, mecanismos envolvidos, potenciais alvos terapêuticos e melhora da qualidade de vida, em um aspecto translacional. Dentre essas neurotoxinas estão a 6-OHDA, paraquat, reserpina, 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) e a rotenona. A 6-OHDA é

captada pelos neurônios dopaminérgicos através do transportador de dopamina (DAT) sendo então oxidada, produzindo peróxido de hidrogênio (H_2O_2) e para-quinona, induzindo assim a morte neuronal através da produção de espécies reativas de oxigênio (ERO). O paraquat, por outro lado, é captado pelos neurônios dopaminérgico com o auxílio de um transportador de aminoácidos neutros, catalisando a formação de ERO através de dois mecanismos: mediada pela indução de ciclo redox e ativando enzimas como NADPH oxidases. A reserpina age inibindo o transportador vesicular de monoaminas (VMAT2), impedindo o armazenamento de DA, que é então metabolizada no citoplasma, gerando ERO e quinonas reativas que resultam em estresse oxidativo e morte celular. O MPTP e a rotenona, como compostos lipofílicos, atravessam facilmente a barreira hemato-encefálica. O MPTP é então metabolizado em 1-metil-4-fenilpiridina (MPP^+) pela enzima monoamina-oxidase B (MAOB), sendo captado nos neurônios dopaminérgicos através do DAT. O MPP^+ e a rotenona acumulam-se nas mitocôndrias onde inibem o complexo I da cadeia respiratória mitocondrial. Essa inibição leva a uma diminuição dos níveis de ATP, um aumento de produção de ERO e consequente morte celular (BOVÉ; PERIER, 2012; LEÃO et al., 2015).

Estudos propõem a administração intranasal de MPTP, no intuito de promover uma degeneração dopaminérgica mais progressiva, mimetizando a DP (PREDIGER et al., 2006, 2009). O modelo intranasal foi o primeiro a apresentar uma relação mais direta entre olfação e a via nigroestriatal num modelo animal de DP, A rotenona, por sua vez, apresenta basicamente os mesmos mecanismos de neurotoxicidade que o MPTP, ou seja, inibe o complexo I da cadeia respiratória mitocondrial, gerando morte neuronal principalmente por apoptose. Porém, ela está associada a uma importante condição de exposição ambiental, haja vista que é utilizada amplamente como pesticida (BETARBET et al., 2006). Dessa forma, vários casos de Parkinsonismo decorrentes dessa exposição já foram relatados na literatura, o que confere uma maior validade ao modelo animal (BETARBET et al., 2000; DHILLON et al., 2008). Ainda, sabe-se que o modelo de rotenona intranigral é efetivo para mimetizar estágios iniciais da DP (MOREIRA et al., 2012; DOS SANTOS et al., 2013; RODRIGUES et al., 2014; AURICH et al., 2017) e, nesse sentido, distúrbios olfatórios, já que a hiposmia tem sido relacionada à degeneração nigrostriatal inicial (SIDEROWF et al., 2005).

1.4 CAFEÍNA E NICOTINA NA DOENÇA DE PARKINSON

A cafeína participa da classe das metilxantinas, consideradas os estimulantes no sistema nervoso central mais consumidos mundialmente, presentes em energéticos, chás, cafés e também em alguns medicamentos indicados para cefaleia e supressores de apetite (ARANDA et al., 1977; NEHLIG; DAVAL; DEBRY, 1992). A cafeína é um inibidor de fosfodiesterases e que possui propriedades lipofílicas, o que permite atravessar a barreira hematoencefálica (MCCALL; MILLINGTON; WURTMAN, 1982; FREDHOLM et al., 1999). Contudo, a hipótese mais aceita para explicar o mecanismo de ação da cafeína no organismo é através do antagonismo não seletivo dos receptores adenosinérgicos, frente uma dose comum diária de café (NEHLIG; DAVAL; DEBRY, 1992), sendo os receptores A1 e A2 capazes de modular a liberação de diferentes neurotransmissores, como por exemplo, a DA (HADFIELD, 1997; ROSS; PETROVITCH, 2001; PREDIGER, 2010).

A nicotina é uma amina terciária encontrada no tabaco, e tem sua ação deflagrada pela ligação a receptores colinérgicos nicotínicos (nAChRs). Estes receptores são distribuídos difusamente em todo o encéfalo. No BO, particularmente, estão restritos a CG e camada mitral (HILL et al., 1993; LE JEUNE et al., 1995, 1996). A ativação do nAChR modula a liberação de neurotransmissores, como acetilcolina, noradrenalina, DA, serotonina, entre outros (BENOWITZ, 1996).

A associação da cafeína e da nicotina com a DP é devida a existência de uma relação inversa, descrita na literatura, entre o consumo de cafeína ou uso de tabaco, e o risco de desenvolvimento da doença, na população mundial (ROSS et al., 2000; SÄÄKSJÄRVI et al., 2008; LI et al., 2015; YANG et al., 2016). Além desses estudos epidemiológicos, há diversas evidências produzidas a partir de modelos animais da DP demonstrando efeitos benéficos da cafeína frente ao comprometimento motor, na diminuição de mediadores neuroinflamatórios, diminuição da depleção dopaminérgica no estriado, redução da degeneração de neurônios TH-ir, aumento do número de neurônios dopaminérgicos e restauração parcial da SNpc (XU et al., 2002, 2016; HSU; WANG; CHIU, 2010; MACHADO-FILHO et al., 2014; SOLIMAN; FATHALLA; MOUSTAFA, 2016). Portanto, sugere-se que essas substâncias possuam um significativo potencial neuroprotetor (ASCHERIO et al., 2001; XU et al., 2002, 2016; TAKEUCHI et al., 2009; LIU et al., 2012). Sugere-se também que os mecanismos relacionados a eles sejam mediados pela indução de fosforilação da

enzima TH, aumentando assim a liberação de DA por esses neurônios no estriado e núcleo accumbens (SOLINAS et al., 2002; BOBROVSKAYA et al., 2007b; DANI; BERTRAND, 2007; GARÇÃO et al., 2013).

Com relação aos distúrbios olfatórios, Prediger e colaboradores (2005) observaram melhora da discriminação olfatória e da memória de reconhecimento social em animais com déficits olfatórios relativos à idade avançada (12 e 18 meses de idade) e que receberam administração de antagonista seletivo A2a ou cafeína. Com isso, puderam demonstrar que o sistema adenosinérgico, portanto, está relacionado ao processamento olfatório (PREDIGER; BATISTA; TAKAHASHI, 2005).

O mecanismo exato pelo qual a cafeína agiria como um antagonista adenosinérgico e melhoraria os sintomas olfatórios na DP ainda não foi bem esclarecido e necessita de mais estudos (PREDIGER, 2010). Porém, parece estar envolvido com um aumento na fosforilação de TH em um resíduo de serina (Ser31) (HSU; WANG; CHIU, 2010). Dessa forma, a cafeína poderia agir semelhantemente no BO modulando, portanto, a função olfatória. A nicotina, por sua vez, possui escassos estudos demonstrando sua ação na olfação (QUIK; WONNACOTT, 2011), porém, em uma avaliação com administração crônica de nicotina, em um modelo hereditário de DP em moscas do gênero *Drosophila*, verificou-se uma reversão dos déficits olfatórios e motores, em comparação com o grupo DP sem nicotina (CHAMBERS et al., 2013).

Com base nos efeitos da cafeína e nicotina em estudos epidemiológicos e pré-clínicos, buscamos investigar os efeitos e mecanismos associados à modulação dos sistemas adenosinérgico e colinérgico diante dos distúrbios olfatórios apresentados em modelo animal da DP induzido por rotenona intranigral.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar os efeitos da modulação dos sistemas adenosinérgico e colinérgico nos distúrbios olfatórios em modelo animal da DP induzido por rotenona intranigral em machos e fêmeas.

2.2 OBJETIVOS ESPECÍFICOS

Avaliar a função locomotora, através do teste de campo aberto, de ratos machos e fêmeas com lesão intranigral tratados com cafeína e/ou nicotina;

Investigar os efeitos da lesão intranigral e tratamentos com nicotina e/ou cafeína na memória olfatória de ratos machos e fêmeas, através do teste de reconhecimento social;

Avaliar os efeitos da administração prolongada de cafeína e/ou nicotina, ou aguda de agonista e antagonista A2a, na discriminação olfatória de ratos machos e fêmeas com lesão intranigral, através do teste de discriminação olfatória;

Avaliar a extensão da lesão intranigral e efeitos dos tratamentos com cafeína e/ou nicotina através de imunohistoquímica para TH na SNpc;

Quantificar os neurônios TH imunorreativos na CG do BO de machos e fêmeas através de imunohistoquímica para TH no BO;

Quantificar a expressão de TH e TH fosforilada no BO de machos e fêmeas através de análises de Western blot no BO.

3 ARTIGO CIENTÍFICO NUMERO 1

Caffeine and nicotine in Parkinson's disease: from olfactory improvement to neuronal restoration.

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Abstract

Parkinson's disease motor disorders reflect a massive degeneration of dopaminergic neurons and dopamine depletion in the nigrostriatal pathway. However, earlier, patients may have olfactory disorders. Studies have shown an inverse relation among consumption of substances containing caffeine or nicotine and the risk of developing PD. The aim of this study was to investigate whether the treatment with caffeine and/or nicotine would be able to revert the olfactory impairment in PD intranigral rotenone animal model and the relation to increased or decreased of OB tyrosine hydroxylase phosphorylation. Male and female Wistar rats (90 days) received intraperitoneal saline, caffeine and / or nicotine for 7 consecutive days after surgery. Seven days after injury, animals from all groups were submitted to behavioral tests. The results indicated that treatment with caffeine and double treatment (caffeine plus nicotine) reversed the olfactory impairment caused by rotenone, in males. In females, olfactory disturbance was not present. Surprisingly, there was no OB TH increased phosphorylation by the caffeine administration, present only in a double treatment. In addition, a trend to increased TH phosphorylation of the nicotine rotenone group was observed, indicating a negative influence of dopamine on olfaction, probably due to its action on OB D2 inhibitory receptors, which may explain the olfactory impairment not reversed by nicotine, observed in the ODT. Besides that, nicotine presented a SNpc dopaminergic neurons restoration for both male and female, indicating a neuroprotective effect against rotenone lesion. In conclusion, caffeine and nicotine presented different beneffits in the rotenone model, improving the olfactory disturbance and reducing the SNpc neuronal death. Addicionally, the intranigral rotenone presented sex differences related to olfactory function, also present in a human population.

Key-words: Parkinson’s disease; olfactory bulb; caffeine; nicotine; sex-dimorphism; dopamine.

Introduction

Non-motor features are present decades before the classical motor disfunction caracteristic of Parkinson's disease (PD) (BRAAK et al., 2003). The reduction of dopaminergic content is responsible for the typical motor signs and symptoms, which arise when more than 50% of the midbrain dopaminergic neurons have degenerated (LANE; DUNNETT, 2008; LANG; LOZANO, 1998). The olfactory disturbances usually appear early in the disease course, mainly as hyposmia/anosmia in aproximatly 90% of the PD patients, negatively impacting their quality of life (DOTY; DEEMS; STELLER, 1988; PONSEN et al., 2004; ROSS et al., 2008). Underlying this pathophysiological condition, it has been intensily discussed in the literature the role of dopamine (DA) as a key participant. However, DA is not the only neurotransmitter related to olfactory processing (DOTY, 2017), leading to a gap for new therapeutic perspectives. Besides, the nigrostriatal degeneration observed in PD dramatically impacts the periglomerular neurons in the olfactory bulb (OB) (ILKIW et al., 2018; RODRIGUES et al., 2014) , indicating a compensatory response due to a nigro-olfactory projection (HÖGLINGER et al., 2015). In view of that, one possible mechanism associated to periglomerular neurons activation would be due to increases in DA biosynthesis/activation by means of boosting tyrosine hydroxylase (TH) enzyme activity (BOBROVSKAYA et al., 2007; KNOWLES; DOUGLAS; BUNN, 2011). Another feasible strategy of modulating such neuronal activation would be by inhibiting phosphodiesterase activity, thus, enhancing the catecholamine synthesis by increasing TH activation (FREDHOLM et al., 1999). Both mechanisms could be reached by the use of nicotine and caffeine, respectively, and with similar potential to improve olfaction. Interestingly, several epidemiological studies have shown that caffeine intake from coffee, tea, energy drinks as well as nicotine exposure from

tobacco smoking present an inverse relation to increased risks of PD (ASCHERIO et al., 2001; YANG et al., 2016).

Another remarkable point that remains unclear is the olfactory performance differences between sexes, particularly in PD. A recent study from our group has suggested that female Wistar rats present a prominent olfactory plasticity related to non-social odors processing, mainly dependent on dopaminergic modulation (RODRIGUES et al., 2018). In fact, male PD volunteers performed significantly worse than female in an odor identification test (LIU et al., 2015). Such sex dymorphism, focusing on how the main olfactory system operates and what are the mechanisms associated to the those differences in PD are still far from being fully understood. Therefore, to our standpoint, is conceivalbe that the activation of dopaminergic periglomerular neurons, within the OB, could be a determinant factor for olfaction, with particular sex-related differences.

Therefore, in the present study, we investigated whether two different pharmacological strategies of promoting increased levels of TH phosphorilation, within the OB, would result in improvements of olfactory performance in an animal model of PD, induced by intranigral rotenone. Additionaly, we sought to identify possible sex-related differences, associated to the treatments, in the olfactory parameters.

Material and Methods

Ethics statement

The experiments were carried out according to the guidelines of Brazilian Guide for Care and Use of Laboratory Animals (COBEA), with recommendations of Federal

University of Paraná (UFPR) and were approved by the Institutional Ethics Committee (approval ID #852).

Animals

Male and female Wistar rats from UFPR bred colony, weighing 250-280 g, were used in the stereotaxic surgery, behavioral tests and cellular/molecular analysis. Male juvenile Wistar rats (30 days) were used to perform the social recognition test (SRT). The animals were randomly housed in groups of five in polypropylene cages with sawdust and maintained in 22±2°C on a 12:12-h light-dark cycle (lights on at 7:00 A.M.) with free access to water and food, except during the behavioral tests.

Experimental design

Before surgeries, the animals were distributed randomly in two groups: sham (n=60 – male; n=60 - female) and rotenone (n=60 – male; n=60 - female). After the surgery procedure, they were redistributed in eight groups (n=15/group): sham saline; sham caffeine; sham nicotine; b.s. (bitartrate salt); sham caffeine + nicotine b.s.; rotenone saline; rotenone caffeine; rotenone nicotine b.s. and rotenone caffeine + nicotine b.s.. On day 0 (Figure 1) a stereotaxic surgery was performed for rotenone SNpc (substantia nigra pars compacta) lesion or vehicle infusion (DMSO – dimetylsulfoxide - to sham groups). Twenty-four hours later (Day 1) started the daily administration of intraperitoneal (i.p.) drugs, which continued for one week, always between 7-9 A.M.). On day 7, thirty minutes after the last intraperitoneal administration, the animals were submitted to behavioral tests and, immediately after, their brains were dissected or perfused for further analysis.

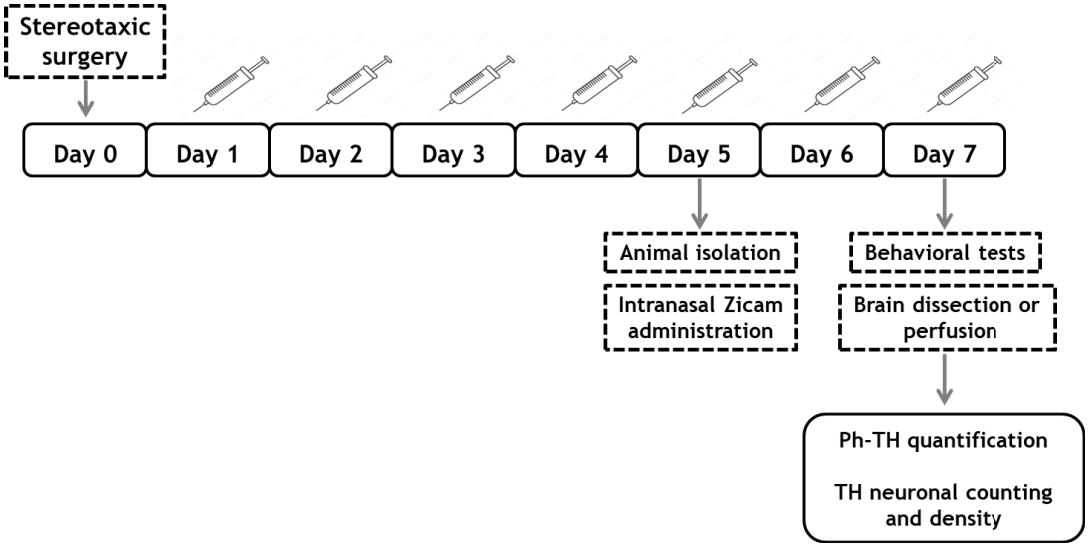

Figure 1. Schematic representation of the experimental design.

Stereotaxic Surgery

The animals were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). All of animals were positioned in the stereotaxic apparatus and the following coordinates were used to the bilateral injury, with bregma as a reference: SNpc (AP) = - 5.0 mm, (ML) = ± 2.1 mm e (DV) = - 8.0 mm (PAXINOS; WATSON, 2005). Needles were guided to the region of interest for a bilateral infusion of 1 µL of rotenone (12 µg/µL) or of DMSO (Sigma-Aldrich®, United States) using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0,33 µL/min for 3 minutes (AURICH et al., 2017; NOSEDA et al., 2014; RODRIGUES et al., 2014; TARGA et al., 2018)

Intraperitoneal drug administration

On the first day after the stereotaxic surgery, the i.p. drugs administration started. According to the group, the animals received 1ml/kg of body weight of saline 0,9%, caffeine anhydrous (10mg/kg) (VETEC code 813), nicotine b. s. (0,5 mg/kg) ((−)-1-

Methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt, Sigma Aldrich, code N5260) or caffeine + nicotine b.s., daily. On day 7, all animals received the last administration 30 minutes before the behavioral tests. Drug doses was selected according to the previous literature reports (GARÇÃO et al., 2013; PREDIGER; BATISTA; TAKAHASHI, 2005).

Intranasal drug administration (zinc gluconate + zinc acetate solution)

The administration of Zicam® Oral Mist (Matrixx Initiatives, Scottsdale, AZ, USA) was performed as previously reported (ILKIW et al., 2018; RODRIGUES et al., 2014). Briefly, the animals were sedated with an intraperitoneal administration of 90 mg/kg ketamine and 3 mg/kg xylazine. Thereafter, approximately 30 mL of Zicam solution was slowly delivered into the nasal cavity using a Hamilton syringe attached to a blunted 30 gauge needle through a polyethylene tube. The polyethylene tube was inserted 15 mm beyond the right and left outer nostrils, first the right nostril, then the left nostril. Part of the solution was expelled through the nostril and dried to allow the animal to continue breathing.

Open Field Test (OFT)

The test evaluated the locomotor performance of each animal and was performed in a circular arena (1 m of diameter x 0.4 m wall) illuminated by one 60 W lamp, thus providing illumination around 100 lx. The animals were placed in the center of the arena and were allowed to freely explore for 5 min. All the tests were video recorded and total distance was computed online by an image analyzer system (Smart junior, Pan Lab, Harvard Apparatus, Spain), which also provides a trajectory travelled by each animal.

Olfactory discrimination task (ODT)

The version used of this task has been modified from (PREDIGER et al., 2006). The apparatus is a box (60 x 40 x 50cm), equally divided into two compartments connected by an open door. Before the test, it was performed an adaptation period to the apparatus of 2 minutes, in which both compartments was with fresh sawdust. After that, clean sawdust is added on one side of the box (non-familiar odor). On the other side of the box, is added sawdust which animals remained isolated for 48 hours before testing (familiar odor). Each animal was placed in the middle of apparatus and video recorded, up to 3 min, to evaluate the investigation time of each compartment. The animal that shows olfactory discrimination impairment tends to explore both compartments equally, indicating absence of discrimination. As a measure of discrimination, an “olfactory discrimination index (ODI)” was calculated by dividing the difference in exploration time between the two compartments (compartment non-familiar - compartment familiar) by the total amount of exploration for both compartments (compartment non-familiar + compartment familiar). ODI was then multiplied by 100 to express it as a percentage of the mean. A positive control for the olfactory impairment was used through the administrion of intranasal Zicam, as previously described.

Social recognition test (SRT)

The SRT was used to assess whether the olfactory memory was affected by the neurotoxin rotenone. The test was adapted from Dantzer and colleagues (DANTZER et al., 1987), and consists of introducing a same specie juvenile animal (intruder) into the housing cage of the forty-eight hours isolated adult animal (resident) to evaluate

the adult animal's interaction with the intruder, for 5 minutes. After the first presentation, the intruder is removed from the cage and waits, isolated, for 20 minutes until the next presentation. The same intruder is then presented for the second time to the same resident and is quantified the interaction for 5 minutes. In the end, the exploration time between the first and the second presentation is calculated. An animal with an impaired social recognition memory interacts with the intruder for a similar time in both presentations. The animal without olfactory memory impairment interacts for less time in the second presentation, recognizing the intruder.

TH- immunohistochemistry within the SNpc and OB

Animals were deeply anesthetized with ketamine immediately after the behavioral tests and were intracardially perfused with saline, then with 4% of the formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and 48 h immersed in fixative solution at 4ºC. The samples were immersed in 30% sucrose solution for 3 days and finally freozen at -80ºC. Sections (40 µm) were obtained from the OB (+7.56 mm and +7.08 mm) and from SNpc (-4.92 mm and -5.28 mm, an interval of 360µm) (PAXINOS; WATSON, 2005; RODRIGUES et al., 2014). The sections were incubated with primary mouse anti-TH antibody (1:1000 - SNpc; 1:8000 - OB; Sigma Aldrich, Missouri, USA – code T2928) prepared in phosphate-buffered saline containing 0.3% Triton X-100 overnight at 4°C. Biotin-conjugated secondary antibody incubation (1:200 anti-mouse - Vector Laboratories, USA), was performed for 2h at room temperature. After several washes in phosphate-buffered saline, antibody complex was localized using the ABC system (Vectastain ABC Elite kit, Vector Laboratories, USA) followed by 3,3´- diaminobenzidine reaction with nickel

enhancement. Cell counts for the glomerular layer of the OB were carried out by the software Image-Pro Express 6, and neuronal density for the SNpc was performed with the software Image J 1.47v. The mean number or density of TH-ir neurons in each hemisphere was considered to be representative of the OB and SNpc neuronal cells in each animal. For each group, a mean value was calculated (percentage relative to the sham control), and compared with those of the other groups. The images were obtained through the use of a motorized Axio Imager Z2 microscope (Carl Zeiss, Jena, DE), equipped with an automated scanning VSlide (Metasystems, Altlussheim, DE).

Western blot analysis

To determine TH and phosphorylated form of TH (p-TH) within the OB, after behavioral tests, animals were decapitated, and brains were rapidly ice-removed and OB were dissected. Until processed for analysis, tissues were stored at -80ºC. Samples were sonicated in lysis buffer containing 150mM NaCl, 50mM Tris-HCl, 2mM EDTA, 1% Triton X-100, 1mM PMSF, 1mM sodium orthovanadate (Na3VO4), 1mM sodium fluoride (NaF) and complete protease and phosphatase inhibitor mixture EDTA-free (Roche). After centrifugation (10 min, 12,000xg, at 4°C) supernatant was collected, and protein concentration was determined by the Bradford method (Bio-Rad, Germany). Samples (60µg) were subjected to SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis – 1mm) and proteins transferred to a nitrocellulose membrane (GE Healthcare). The membranes were then blocked in 2% BSA diluted in TBS-T (blocking solution) for 1h at room temperature and incubated overnight with the desired antibody diluted in blocking solution. The antibodies used were: mouse monoclonal anti-GAPDH (code SC-

32233, Santa Cruz), anti-TH (code B2409, Santa Cruz), anti-phosphorylated-Tyrosine Hydroxylase (p-TH) ((Ser40) code 2791, Cell Signaling). After primary antibody incubation, membranes were extensively washed with TBS-T and incubated with HRP-conjugated secondary antibody (Sigma Aldrich) in blocking solution for 1h at room temperature. After p-TH incubation, membranes were stripped and immunobloted against TH. Finally, membranes were washed again and immune complexes were detected using the ECL chemiluminescent detection system (GE Healthcare Life Sciences, Brazil). The protein levels were quantified by densitometry using ImageJ v1.47 software (National Institutes of Health, USA).

Statistical analysis

The ODT and SRT were analysed by three-way repeated measures ANOVA (factors: lesion; treatment and compartment for ODT; lesion, treatment and presentation for SRT) followed by Bonferroni´s post hoc test. The OFT, ODI, TH-immunohistochemistry and Western blot quantifications were analyzed by two-way ANOVA (factors: lesion and treatment; or lesion and sex differences for male X female comparisons) followed by Bonferroni´s post hoc test. Spearman's correlation coefficient (r) was calculated to establish correlations between molecular and histochemical parameters. Values were expressed as mean (for the ODI) or mean ± standard error of the mean (SEM). Significant differences were set at $P \leq 0.05$. For groups distribution representative visualization, mean values were plotted in a scatter diagram (Cartesian coordinates), comparing behavioral, molecular and histochemical parameters.

Results

Open field test (OFT)

Among male rats (Figure 2A), a hyper locomotion was observe, by sham caffeine group comparing to sham saline ($P<0.05$) and sham nicotine ($P<0.01$) groups. The rotenone caffeine + nicotine group traveled a significantly higher distance compared to all the other injured groups ($P<0.05$) and also compared to the sham with the same treatment ($P<0.05$). The analysis indicated the influence of the treatment effect [$F(3.83)=8.13$; $P<0.001$], with the influence of the lesion factor [$F(1.83)=4.63$; $P=0.034$] and also with a significant interaction effect [$F(3.83)=6.70$; $P=0.0004$]. Representative trajectories of the male groups are shown in the Figure 2B.

In the females OFT (Figure 2C), the locomotor activity of the rotenone saline group was impaired compared to the sham saline group ($P<0.05$), demonstrating the lesion factor effect [$F(1.105)=13.48$; $P=0.0004$]. However, no significant treatment effect [$F(3.105)=2.42$; $P=0.07$] or interaction [$F(3.105)=1.901$; $P=0.13$] were observed. Representative trajectories of the female groups are shown in the Figure 2D.

Comparing male and female sham/rotenone saline groups (Figure 2E) was observed that female sham group had a naturally higher locomotion than male sham group ($P < 0.01$) and female rotenone group ($P < 0.001$). On the other hand, male rotenone group presented a higher distance traveled than female rotenone group ($P < 0.001$). Those traveled distance differences demonstrate the significant interaction among lesion and sex factors [$F (1.47)=31.56$; $P<0.0001$] in the OFT.

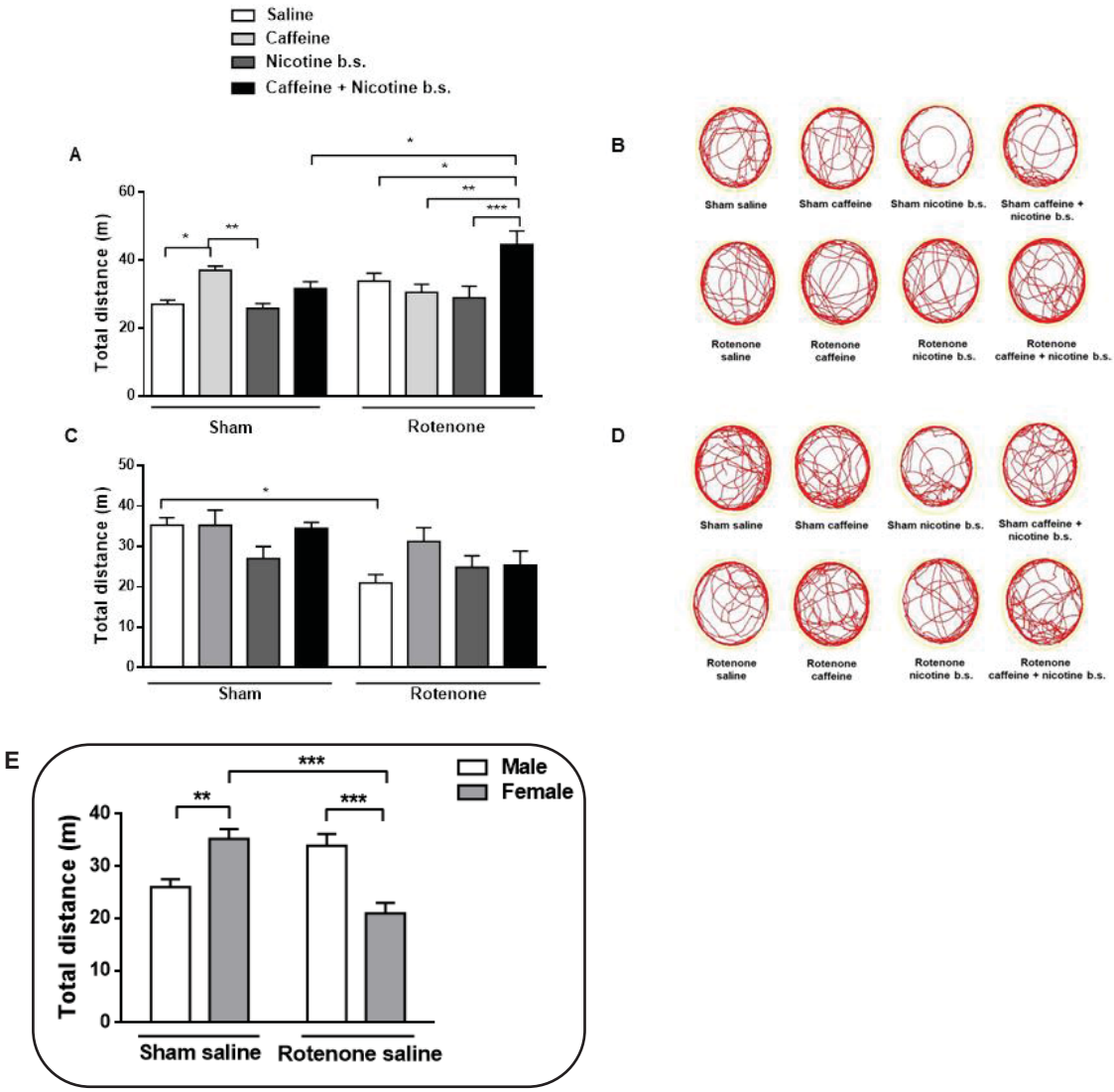

Figure 2. Open field test (OFT). Distance travelled (m) in the OFT 7 days after surgery. **A.** Performance of male groups in the OFT. **B.** Representative trajectories of male rats. **C.** Performance of female groups in the OFT. **D.** Representative trajectories of female rats. **E.** Comparison among male and female saline groups in the OFT. The bars are represented as mean ± standard error of the mean. N=9-15/group, *P≤0.05, **P≤0.01, ***P≤0.001, comparing the means of the travelled distance between groups. Two-way ANOVA followed by Bonferroni post hoc test.

Olfactory discrimination task (ODT) and olfactory discrimination index (ODI)

Analyzing the ODT, male rats of sham saline ($P<0.01$) and sham caffeine ($P<0.001$) groups had a significant olfactory discrimination, spending more time in the familiar odor compartment (Figure 3A). A similar result was observed in the rotenone caffeine group ($P<0.001$), indicating an olfactory discrimination improvement, without lesion effect [$F(1.93)=0.98$; $P=0.32$]. On the opposite, the other rotenone groups present an impaired discrimination in this task, similar to the sham nicotine b.s and caffeine plus nicotine b.s groups and also to the zicam group (positive control to olfactory discrimination impairment). No treatment effect was observed [$F(3.93)=0.20$; $P=0.90$] and also any interaction [$F(3.93)=0.92$; $P=0.43$].

In a different perspective, the ODI obtained for male rats showed that mean values closest to zero represent worse olfactory discrimination index (Figure 3B). The analysis indicated the occurrence of a treatment effect associated to the intraperitoneal caffeine administration [$F(3.89)=4.80$; $P=0.003$], without the influence of the lesion factor [$F(1.89)=0.74$; $P=0.39$] and no significant interaction factor [$F(3.89)=1.41$; $P=0.24$].

On females, in opposite from males, rotenone did not cause any olfactory discrimination impairment, for any group ($P<0.05$), including zicam group (Figure 3C). Animals for all groups spent more time in the familiar odor compartment, especially those with nicotine b.s. or caffeine plus nicotine b.s. administrations, indicated by a treatment effect [$F(3.92)=1.90$; $P<0.001$]. However, no lesion effect was observed [$F(1.92)=1.18$; $P=0.28$] and no interaction also [$F(3.92)=2.61$; $P=0.056$]. The female ODI (Figure 3D) showed no significant differences between groups, indicating the same unaltered olfactory discrimination observed in the ODT.

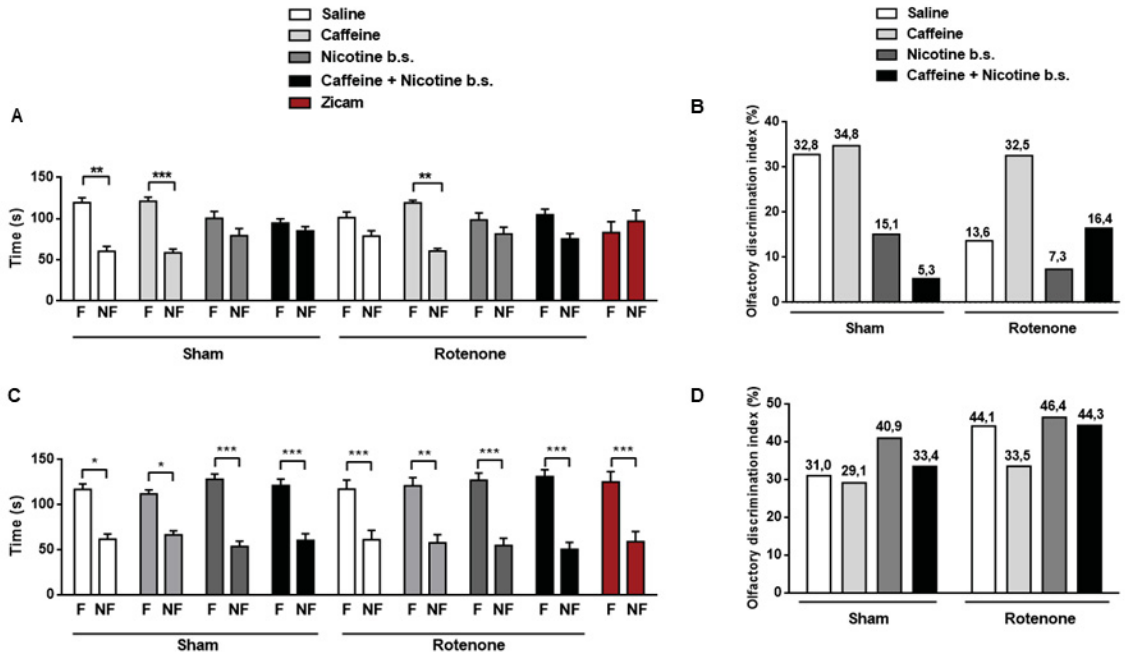

Figure 3. Olfactory discrimination task (ODT) and olfactory discrimination index (ODI). Time (s) spent in familiar (F) and non-familiar (NF) odors compartments in the olfactory discrimination task (ODT) 7 days after surgery. **A**. Male groups olfactory performance in the OFT. **B**. Males ODI. **C**. Female groups olfactory performance in the OFT. **D**. Female ODI. The bars are represented as mean ± standard error of the mean (ODT) or as group's mean (ODI). ODI was calculated by (NF-F/NF+F)*100, NF is the time spent in the compartment with non-familiar odor and F is the time spent in the compartment with familiar odor.N=10-15/group, *P≤0.05, **P≤0.01, ***P≤0.001. Three-way ANOVA followed by Bonferroni post hoc test (ODT) and wo-way ANOVA followed by Bonferroni post hoc test (ODI).

Social recognition test (SRT)

The SRT was performed to evaluate the olfactory memory in the rotenone model. In male rats (Figure 4A), except for the sham nicotine b.s. and rotenone caffeine plus

nicotine b.s. groups, all the other presented an intact olfactory memory ($P<0.05$), with a higher exploration time in the presentation 1. Lesion effect was found [$F(1.72)=7.86$; $P=0.006$] but without treatment [$F(3.72)=2.01$; $P=0.12$] and interaction [$F(3.72)=2.58$; $P=0.06$] factors.

Female rats had an olfactory memory impaired only for rotenone saline group, however, without significant lesion effect [$F(1.97)=0.003$; $P=0.95$]. The other groups had an elevated exploration time in the first presentation compared to the second one ($P<0.05$). Treatment [$F(3.97)=1.41$; $P=0.24$] and interaction [$F(3.97)=2.40$; $P=0.07$] effects were not observed.

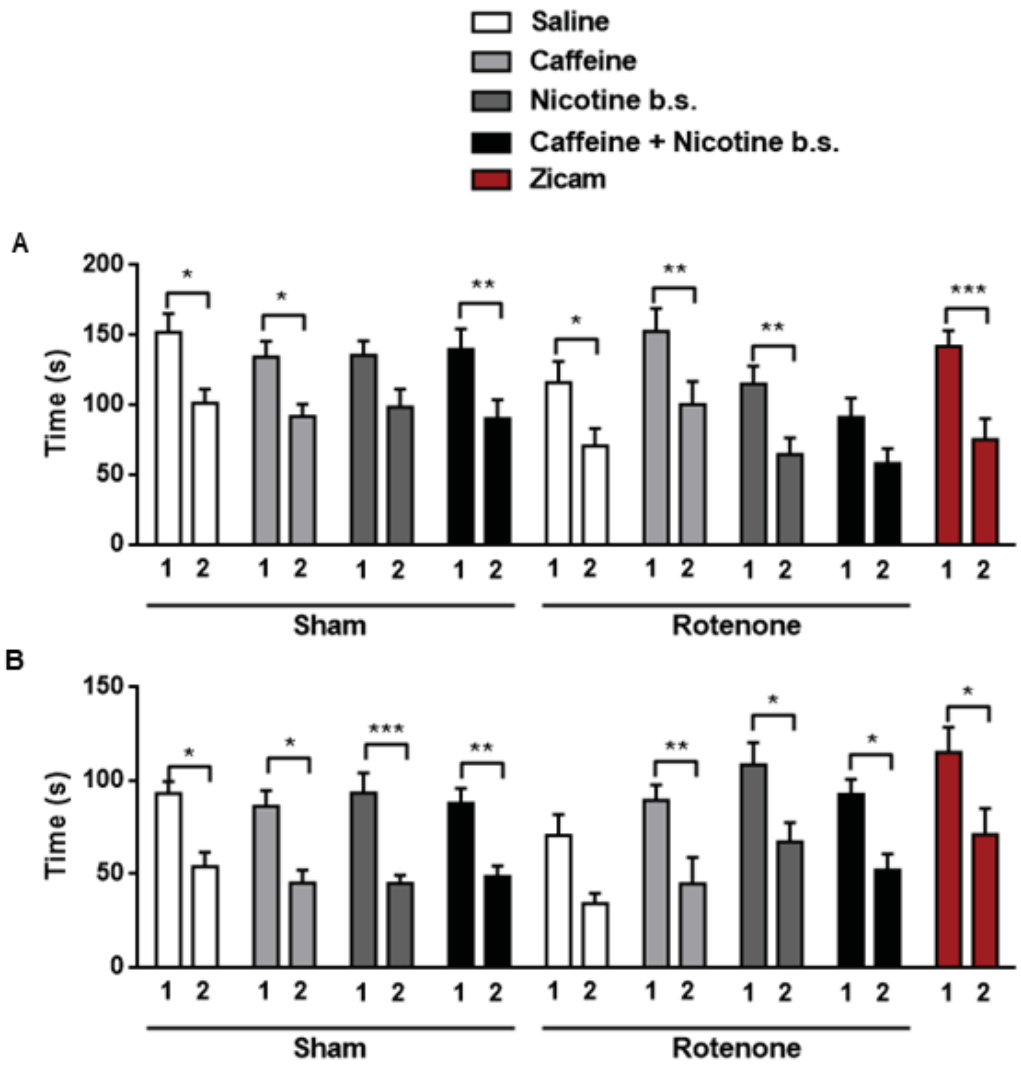

Figure 4. Social recognition test (SRT). Time (s) that resident animal spent exploring the intruder animal in the first (1) and second (2) presentations. **A**. Interaction time observed in male rats during the SRT. **B**. Interaction time observed in female rats during the SRT. The bars are represented as mean ± standard error of the mean. N=10-15/group, *P≤0.05, **P≤0.01, ***P≤0.001. Three-way ANOVA followed by Bonferroni post hoc test.

TH-immunohistochemistry within the SNpc and OB

Evaluating the TH-ir optical density within the SNpc in males (Figure 5A-B), was observed a significant lesion (90%) comparing the sham saline and rotenone saline

groups (P<0.05), evidenced by a lesion effect [F(1.61)=18.83; P<0.0001]. Surprisingly, this lesion was attenuated by nicotine b.s (P<0.01) and caffeine plus nicotine b.s. (P<0.001) treatments. The sham caffeine plus nicotine b.s. group had an elevated TH-ir neuronal density compared to sham saline group (P<0.001). There were a significant treatment effect [F(3.61)=12.99; P<0.0001] and interaction [F(3.61)=2.25; P=0.09].

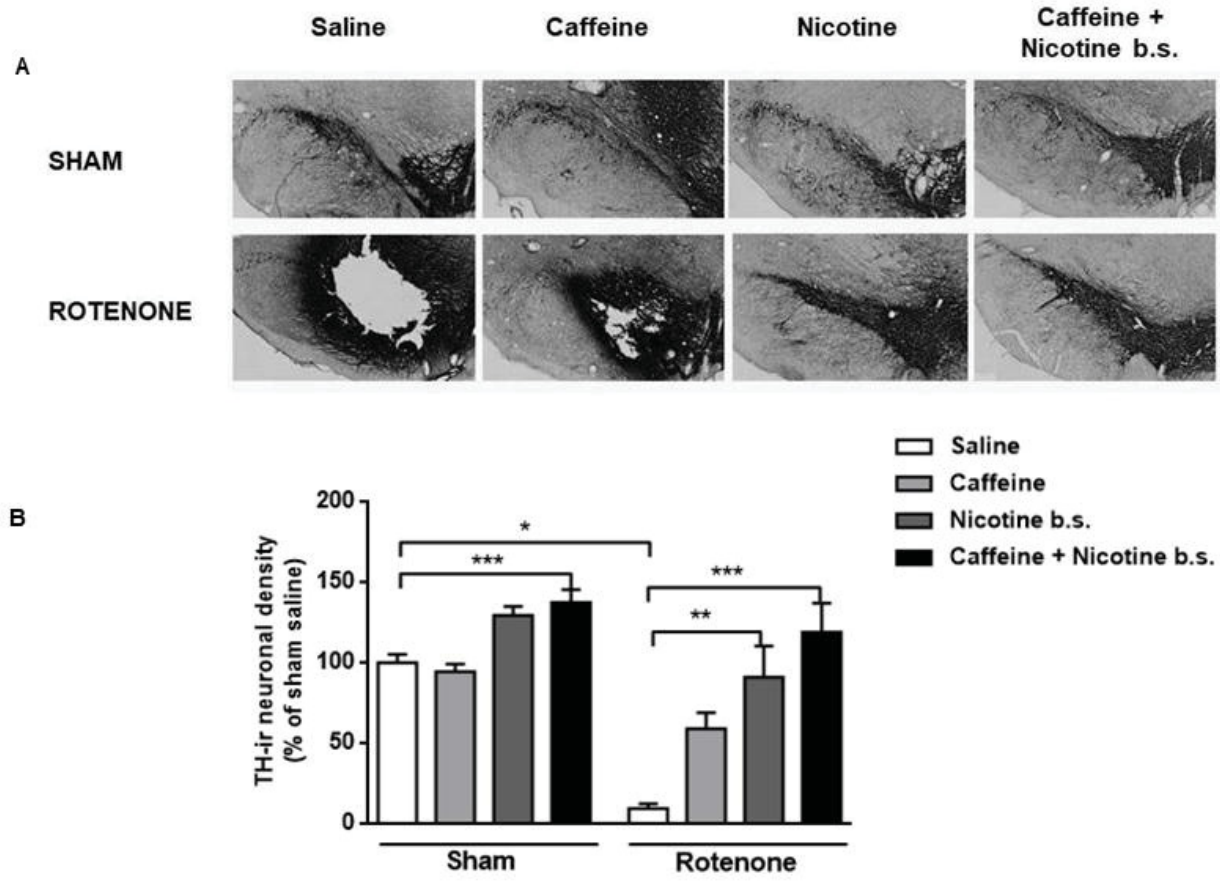

Figure 5. Immunohistochemistry of TH-ir neurons in the SNpc from male rats**. A.** Representative images of dopaminergic neurons within the SNpc (magnification 20x). **B.** SNpc neuronal density analysis. The bars are represented as mean ± standard error of the mean, N=3/group, *P≤0.05, **P≤0.01, ***P≤0.001. Two-way ANOVA followed by Bonferroni post hoc test.

Similarly to male, female also present a significant, but less prominent, rotenone injury comparing the sham saline and the rotenone saline groups (P<0.001), the caffeine treated groups (P<0.001) and the caffeine plus nicotine b.s. treated groups (P<0.001), also demonstrated by a lesion effect [F(1.84)=152.5; P<0.0001] (Figure 6A-B). Was demonstrating a higher TH-ir density in the rotenone nicotine group, compared to rotenone saline (P<0.001), rotenone caffeine (P<0.001) and rotenone caffeine plus nicotine b.s. (P<0.05), with a significant treatment effect [F(3.84)=7.40; P=0.0002] and interaction [F(3.84)=7.39; P=0.0002].

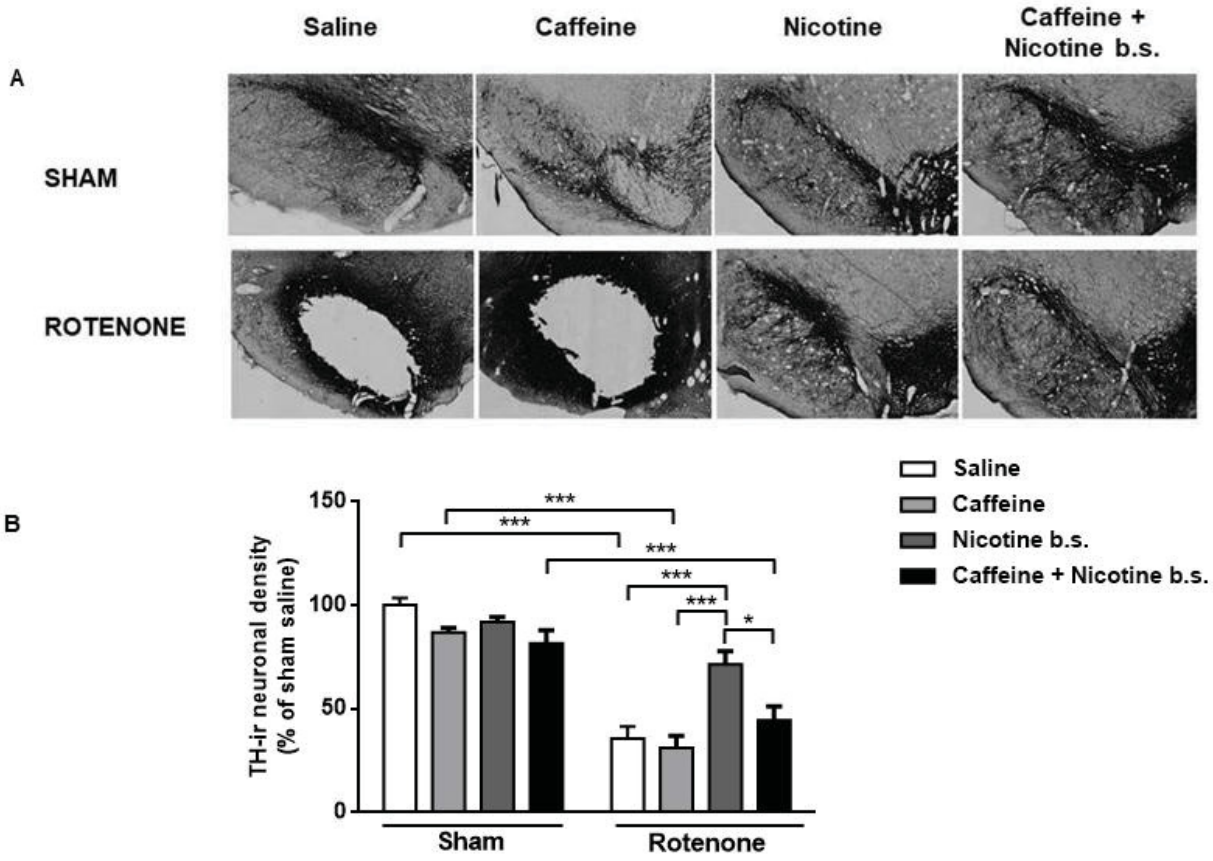

Figure 6. Immunohistochemistry of TH-ir neurons in the SNpc from female rats**. A.** Representative images of dopaminergic neurons within the SNpc (magnification 20x). **B.** SNpc neuronal density analysis. The bars are represented as mean ± standard

error of the mean, N=3/group, *P≤0.05, ***P≤0.001. Two-way ANOVA followed by Bonferroni post hoc test.

Comparing male and female SNpc TH-ir densities, with saline treatment (Figure 7A), was observed that female sham group showed an increased neuronal density compared to male sham group ($P<0.01$), with significant sex difference effect [$F(1.28)=9.05$; $P=0.005$], but no significant changes with rotenone injured groups. Lesion effect was present [$F(1.28)=71.4$; $P<0.0001$], and the interaction [$F(1.28)=4.66$; $P=0.04$] also.

Similar result was found with caffeine treatment (Figure 7B), demonstrated by a higher TH-ir density in the female sham group compared to male sham group ($P<0.05$). For rotenone injury, opposite results were shown: male rotenone group had increased TH-ir density compared to female rotenone group ($P<0.01$). However, no significant sex difference effect was demonstrated [$F(1.42)=0.001$; $P=0.97$], but significant lesion effect [$F(1.42)=65.30$; $P<0.0001$] and interaction [$F(1.42)=21.67$; $P<0.0001$].

For nicotine b.s. treatment (Figure 7C), no sex differences was observed regarding SNpc TH-ir density [$F(1.38)=2.86$; $P=0.09$]. Lesion factor was significant [$F(1.38)=12.78$; $P=0.001$], but there was no interaction [$F(1.38)=0.15$; $P=0.70$].

The rotenone male group had a higher TH-ir density compared to female rotenone group ($P<0.01$), both caffeine plus nicotine b.s treated (Figure 7D). Accordingly, sex difference effect was found [$F(1.39)=7.93$; $P=0.007$]. Lesion [$F(1.39)=13.04$; $P=0.0009$] and interaction [$F(1.39)=5.53$; $P=0.02$] factors were present.

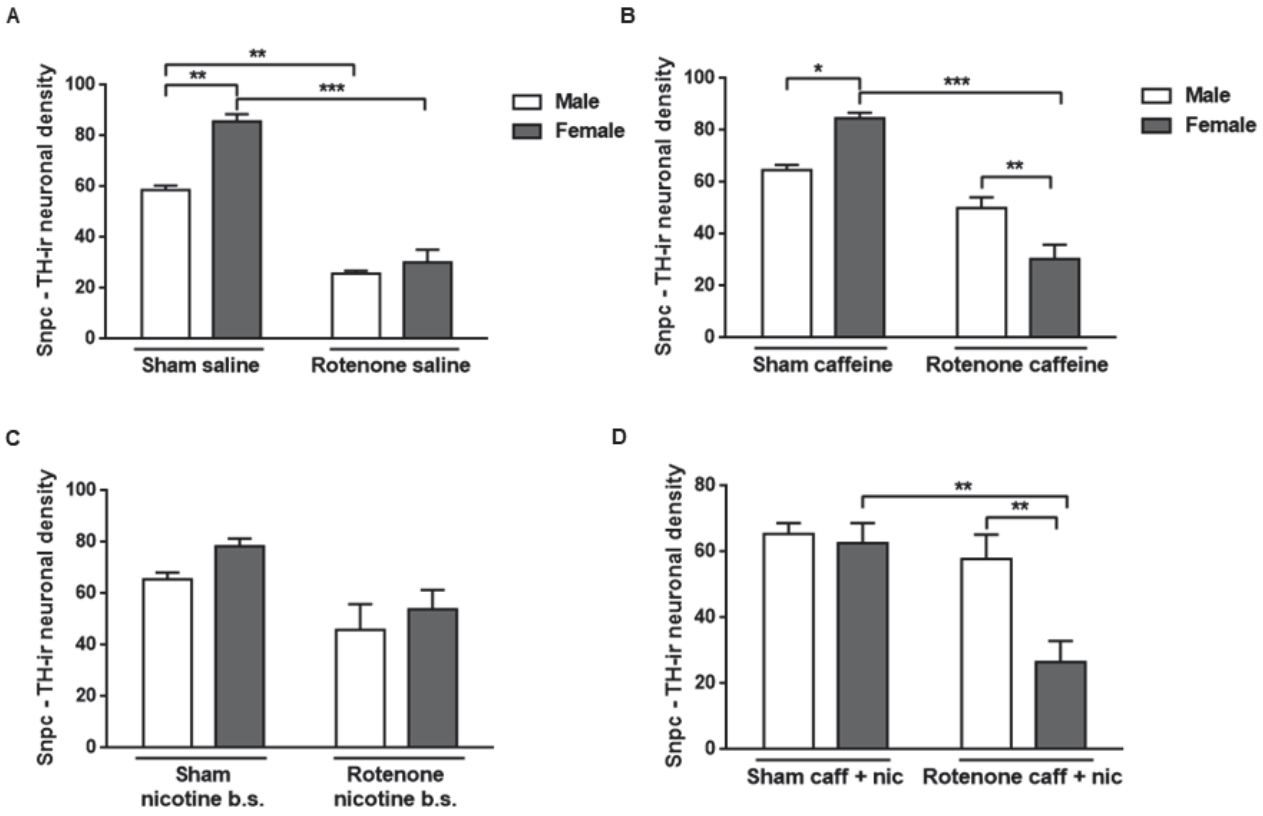

Figure 7. Comparison among male and female treated groups concerning SNpc neuronal densities. **A.** Saline groups; **B.** Caffeine groups; **C.** Nicotine groups and **D.** Caffeine + nicotine groups. The bars are represented as mean ± standard error of the mean. N=3/group, *P≤0.05, **P≤0.01, ***P≤0.001. Two-way ANOVA followed by Bonferroni post hoc test.

Analyzing male OB, neuronal counting reveled an increased number of TH-ir neurons in the GL of the sham nicotine b.s. group compared to sham saline (P<0.01), sham caffeine (P<0.01) and sham caffeine plus nicotine b.s. groups, associated with a significant treatment effect [F(3.52)=10.1; P<0.0001] (Figure 8A-B). Lesion effect was present either [F(1.52)=5.09; P=0.03], but no differences comparing the rotenone groups was found. There was no significant interaction between effects [F(3.52)=2.28; P=0.09].

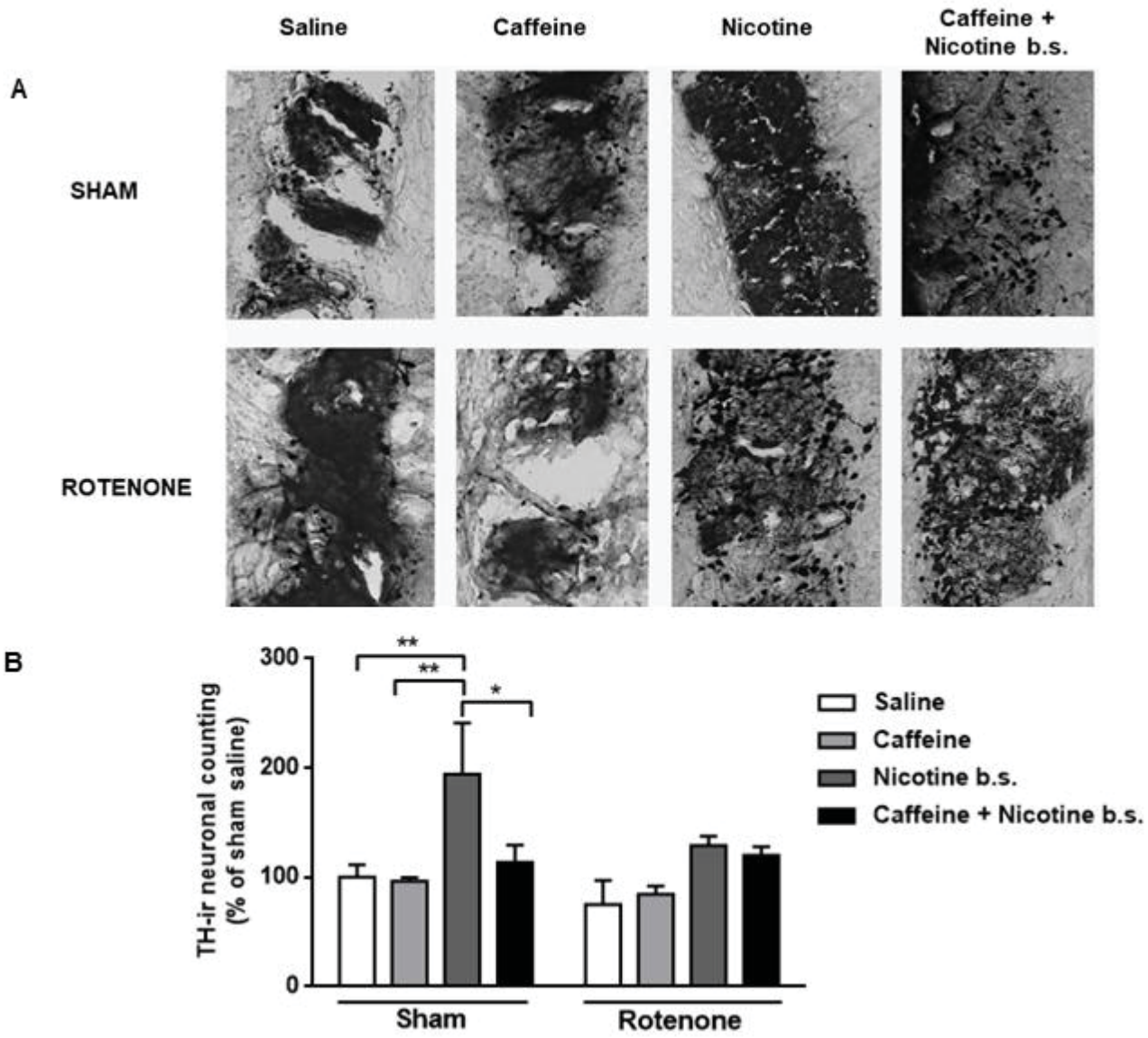

Figure 8. Percentage of TH-ir neurons in the glomerular layer within the OB of male rats **A.** Representative images of dopaminergic neurons within the OB (magnification 50x). **B.** Corresponds to OB neuronal counting analysis. The bars are represented as mean ± standard error of the mean, N=3/group, *P≤0.05, **P≤0.01. Two-way ANOVA followed by Bonferroni post hoc test.

The number of TH-ir neuron in the female OB GL was similar among groups (Figure 9A-B), without treatment effect [F(3.78)=1.041; P=0.3790], but with significant lesion

effect [F(1.78)=4.576; P=0.0356]. However, no interaction was found [F(3.78)=1.459; P=0.2322].

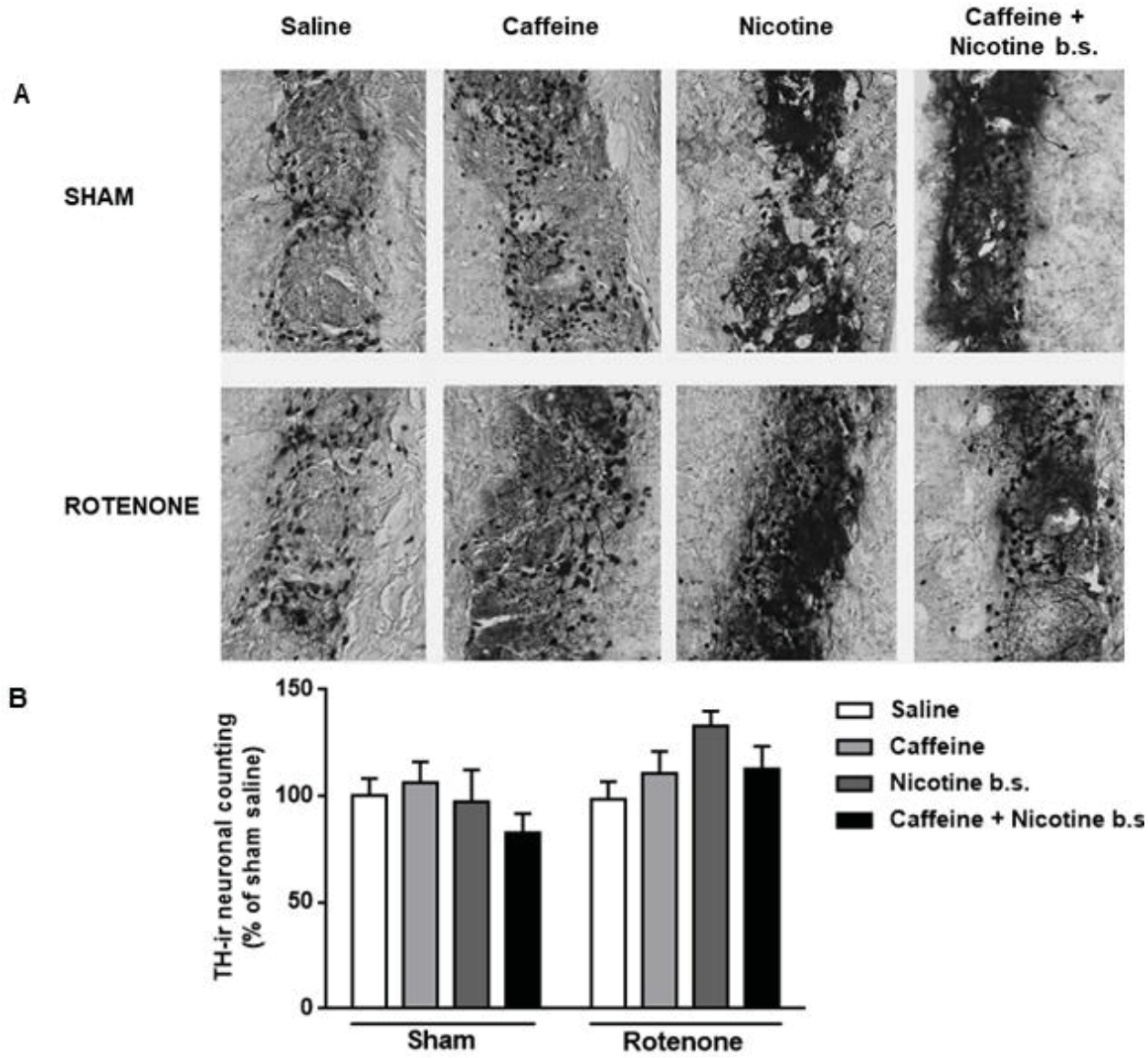

Figure 9. Percentage of TH-ir neurons in the glomerular layer within the OB of female rats. **A.** Representative images of dopaminergic neurons within the OB (magnification 50x). **B.** Corresponds to OB neuronal counting analysis. The bars are represented as mean ± standard error of the mean, N=3/group. Two-way ANOVA followed by Bonferroni post hoc test.

Comparing male and female OB TH-ir neurons by treatment (Figure 10 A-D), only nicotine administration caused an increased GL TH-ir cells number in the male sham group compared to female sham group (P<0.01), with significant sex difference effect [F(1.28)=8.66; P=0.006] (Figure 10B). The rotenone injury produce similar GL neurons quantification for male and female, without lesion effect [F(1.28)=0.88; P=0.35], however, with a significant interaction [F(1.28)=10.15; P=0.003].

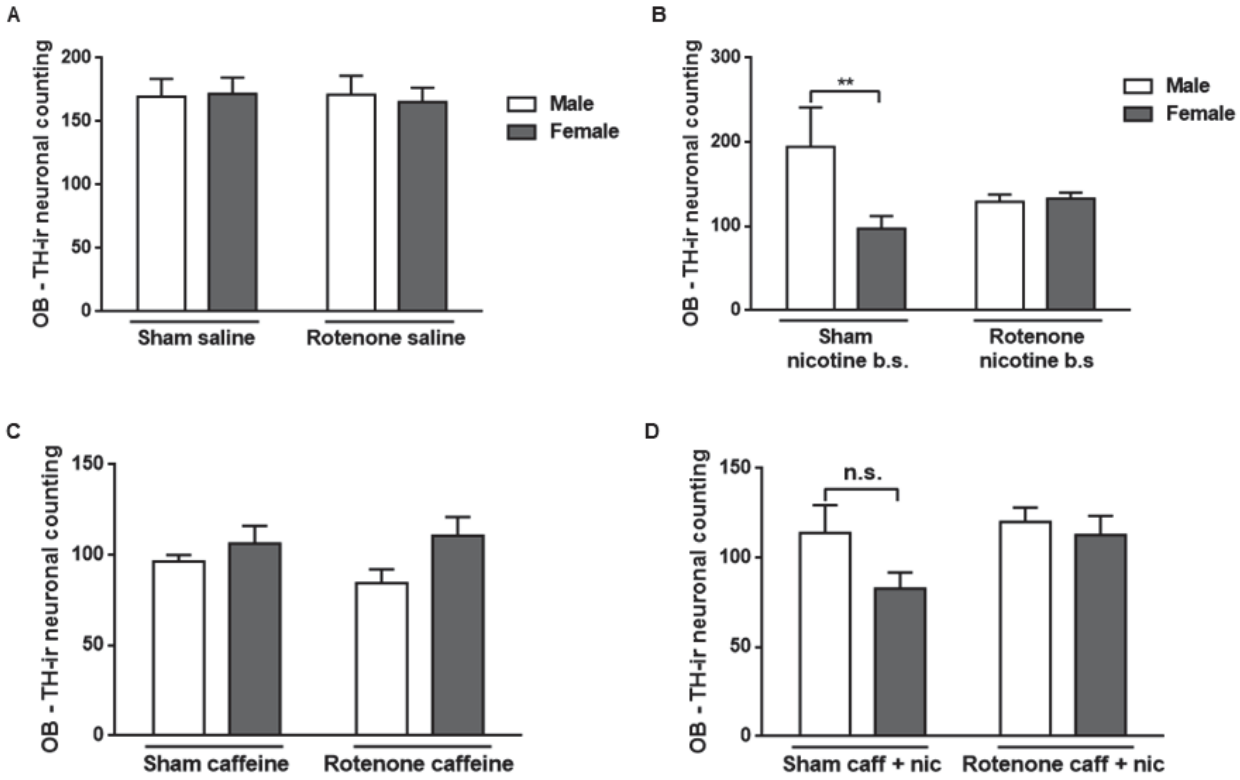

Figure 10. Comparison among male and female treated groups concerning OB neuronal counts. **A.** Saline groups; **B.** Caffeine groups; **C.** Nicotine groups and **D.** Caffeine + nicotine groups. The bars are represented as mean ± standard error of the mean. N=3/group, **P≤0.01. Two-way ANOVA followed by Bonferroni post hoc test. N.s.: non-significant difference.

Western blot OB analysis

Male OB p-TH density was investigated and a significant increase was found for sham caffeine plus nicotine b.s. group, compared to sham saline ($P<0.05$) and rotenone caffeine plus nicotine b.s. ($P<0.01$) groups (Figure 11A-B), with influence of treatment [$F(3.16)=8.84$; $P=0.001$], lesion [$F(1.16)=13.79$; $P=0.002$] and interaction [$F(3.16)=4.080$; $P=0.02$] factors. Besides that, TH/GAPDH (not phosphorylated form) (Figure 11C) analysis demonstrated a significant decreased protein density in the rotenone caffeine group, compared to sham caffeine group, associated with lesion effect [$F(1.16)=43.44$; $P<0.0001$]. However, no treatment effect [$F(3.16)=0.8070$; $P=0.51$] or interaction [$F(3.16)=0.69$; $P=0.56$] were found.

Female OB p-TH density was higher for the sham nicotine b.s. group, compared to sham saline group ($P<0.05$), but lower compared to rotenone nicotine b.s. group ($P<0.05$), with influence of lesion effect [$F(1.16)=40.88$; $P<0.0001$] (Figure 11 D-E). A similar result was found comparing sham caffeine plus nicotine b.s and rotenone caffeine plus nicotine b.s. groups ($P<0.001$). Lower p-TH density was observed in the rotenone saline group, compared to nicotine b.s ($P<0.001$) and caffeine plus nicotine b.s. ($P<0.001$) groups, showing a significant treatment effect [$F(3.16)=24.40$; $P<0.0001$] and interaction [$F(3.16)=6.42$; $P=0.004$]. Agreeing with that, rotenone caffeine group present a lower density compared to nicotine b.s ($P<0.01$) and caffeine plus nicotine b.s. groups. Besides that, sham caffeine group presented an increased TH/GAPDH protein density in the OB, compared to sham saline group ($P<0.01$), similar to the caffeine plus nicotine b.s. group ($P<0.05$), influenced by the treatment effect [$F(3.16)=11.05$; $P=0.0004$] (Figure 11F). No lesion effect was found [$F(1.16)=1.54$; $P=0.23$], however, the interaction was present [$F(3.16)=3.55$; $P=0.04$].

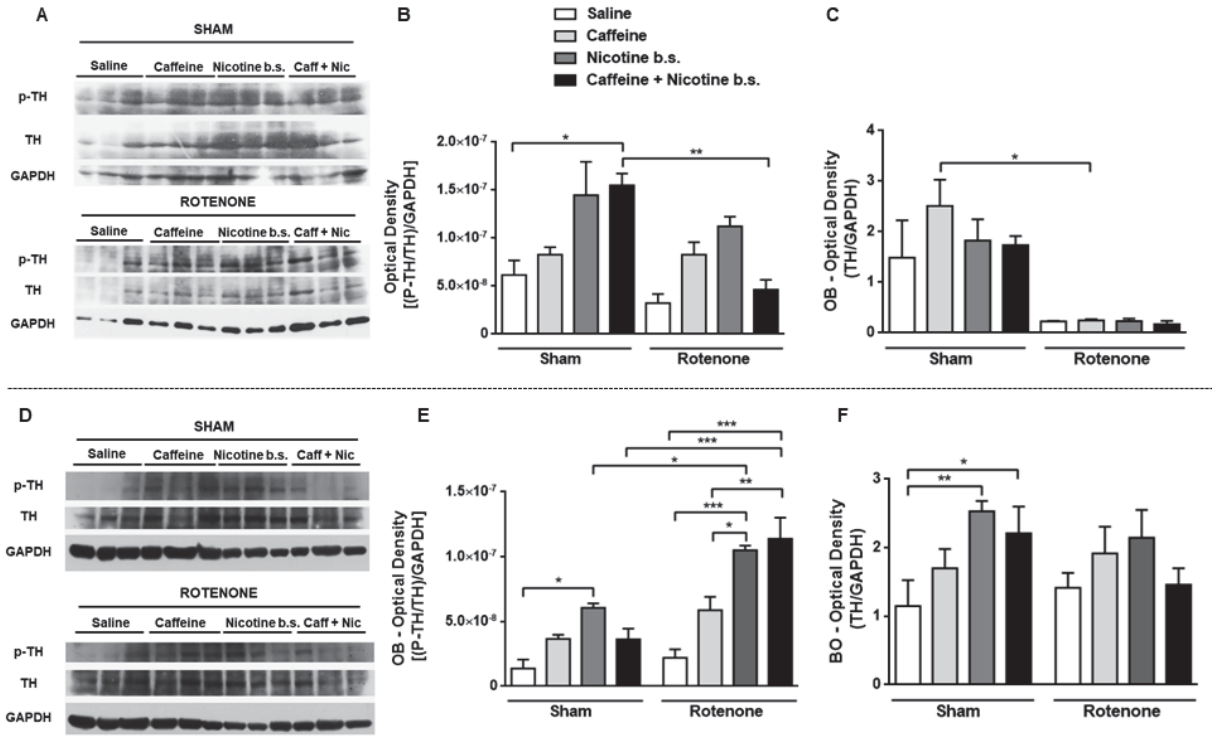

Figure 11. Western blot analysis of normalized p-TH expression within the OB. **A.** Representative images of the bands from male groups; **B.** Optical densities comparision of normalized p-TH expressions, ; **C.** Optical densities comparision of normalized TH expressions; **D.** Representative images of the bands from female groups; **E.** Optical densities comparision of normalized p-TH expressions; **F.** Optical densities comparision of normalized TH expressions. The bars are represented as mean ± standard error of the mean, N=3/group, *P≤0.05, **P≤0.01, ***P≤0.001. Two-way ANOVA followed by Bonferroni post hoc test. P-TH: phosphorylated form of tyrosine hydroxylases; TH: tyrosine hydroxylases; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Comparing male and female p-TH protein density in the OB by treatments (Figure 12A-D), caffeine and caffeine plus nicotine b.s. administrations produced a higher density in sham male group compared to sham female group (P<0.05), with significant sex difference effects [F(1.8)=14.57; P=0.0051] and [F(1.8)=4.400;

P=0.0692] respectively (Figure 12C-D). Besides that, an opposite sex difference was found for rotenone caffeine plus nicotine b.s. groups, with higher phosphorylated protein density in female group, compared to male (P<0.05). No significant lesion effect [F(1.8)=1.450; P=0.2629] or interaction [F(1.8)=1.465; P=0.2607] were found for caffeine treatment. Similarly, lesion effect [F(1.8)=1.627; P=0.2379] wasn't associated with caffeine plus nicotine b.s treatment, however, there was an interaction effect [F(1.8)=58.52; P<0.0001].

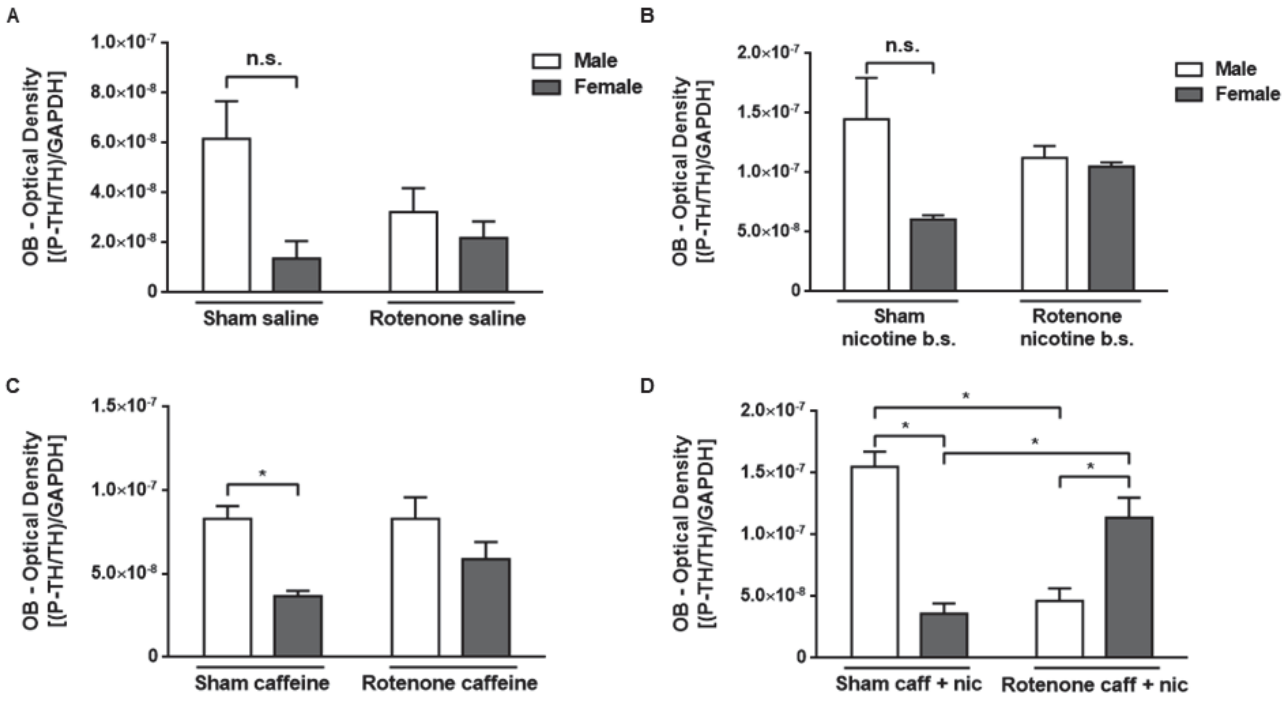

Figure 12. Comparison among male and female treated groups concerning normalized p-TH expression within the OB. **A.** Saline groups; **B.** Nicotine groups ; **C.** Caffeine groups and **D.** Caffeine + nicotine. The bars arerepresented as mean ± standard error of the mean. N=3/group, *P≤0.05. Two-way ANOVA followed by Bonferroni post hoc test. N.S.= non-significant.

Spearman's correlation (Figure 13D) revealed a moderate positive coefficient (r=0.69) between SNpc TH-ir neuronal density and OB p-TH protein expression, however, presenting a barely significant "p" value (P=0.07).

Discussion

The locomotor activity showed in the OFT suggests that males presented a hyperlocomotion caused by caffeine administration (sham group), which is a common effect in this dose for rats (10mg/kg) (MARIN et al., 2011) or similarly in mice (12.5mg/kg) (YACOUBI et al., 2000), even 30 minutes after the administration (HSU; WANG; CHIU, 2010). In fact, in a 6-hydroxydopamine (6-OHDA) PD model, caffeine is also capable to increase the locomotion, reducing the apomorphine-induced rotation (MACHADO-FILHO et al., 2014). Additionally, pre-treatment of caffeine (10 mg/kg/day), during 5 consecutive days, counteracted the locomotor impairment produced by 1-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP) administration in rats (PREDIGER, 2010). On the other hand, in our current study, the intranigral rotenone lesion, in males, did not impact locomotor parameters as previously reported (Rodrigues et al., 2014). However, in female rats, rotenone was able to produce locomotor impairment in comparison to sham saline group. Interestingly, a sex comparison showed that female sham saline group exhibited an increased general activity compared to the respective male group. Besides, female rats appeared to be more affected than male in terms of rotenone-induced locomotor impairment, perhaps because their total distance baseline, for the sham saline, was increased compared to male rats. Considering the caffeine + nicotine treatment, male rats presented a hyperlocomotion, compared to all rotenone groups and also with the sham caffeine + nicotine b.s. group. This result is consistent with a previous report

that demonstrated a locomotor sensitization to nicotine (0,5mg/kg) after an A2a receptor antagonist (ZM241385) injection in rats (Garção et al., 2013). On the opposite, no differences among female sham saline and sham caffeine + nicotine groups were observed.

The rotenone-induced lesion in the SNpc was significantly more severe in males than in females rats. However, it was prevented by nicotine administration in males and females, and also by caffeine + nicotine in male rats, but not in female. In fact, a recent study demonstrated a nicotine-induced neuroprotective effect in a PD rat model inflicted by medial forebrain bundle rotenone injection (1µg/µl) and subcutaneous nicotine injections (1mg/kg, 5 days before injury and 30 days after), thus, increasing the number of SNpc TH-ir neurons compared to rotenone vehicle group (Mouhape et al., 2018). A similar study demonstrated an increase in the TH-ir neurons within the SNpc after a subcutaneous injection of nicotine (0.21 or 0.42 mg/kg daily) 30 min before each oral administration of rotenone (12 mL/kg body weight) (28 days protocol) (Takeuchi et al., 2009). Caffeine partially restored the neuronal density of dopaminergic neurons in male rats (Fig. 5A-B), similarly to the result reported by Soliman and colleagues in their histological analyses with 10mg/kg and 20mg/kg caffeine administrations (12 days) in rats, (Soliman et al., 2016). In addition, 10mg/kg caffeine administration for 2 weeks presented neuroprotective effects by increasing striatal DA content compared to 6-OHDA-injured group (Machado-Filho et al., 2014). Moreover, caffeine (20mg/kg) was able to promote elevated striatal DA content after a typical dopaminergic lesion induced by MPTP (Kui Xu et al., 2002). A comparision between sexes indicated a basal increased density in TH-ir neurons within the SNpc in females compared to males, although the rotenone intranigral exposure revealed a similar level of neuronal lesion in both

sexes. In fact, treatments with caffeine and caffeine + nicotine impacted more drastically the density of TH-ir neurons in males than in female rats, perhaps as a result of the higher basal TH-ir density in female rats.

In a previous study, we demonstrated that SNpc rotenone lesion was able to increase OB TH-ir neurons in male rats, compared to sham control (Rodrigues et al., 2014). However, in the present study, we did not observed an absolute increase induced by rotenone, although, similar densities of OB TH-ir neurons were observed for sham saline and rotenone saline male groups. In female rats the TH-ir neuronal density within the OB did not change for the groups tested, similar to a previous report (Rodrigues et al., 2018).

The ODT showed that caffeine remarkably protected the olfactory function in male rats exposed to a nigrostriatal lesion. Intriguingly, nicotine alone, as well as the association between nicotine and caffeine did not prevent the olfactory impairment caused by rotenone. The mechanism underlying this process is not clear, however, the hypothesis proposed here concerns the phosphodiesterase inhibition and the consequent increase in TH phosphorilation in the OB, thus, leading to an enhanced periglomerular neuronal activity and consequent improvements in the olfactory processing. Indeed, western blot analysis revealed that OB p-TH protein expression, in sham male rats, is increased, particularly as a result of the association between nicotine and caffeine. However, nigrostriatal lesion induced by rotenone was able to mitigate such increased phosphorylation, as well as negatively impacting TH expression for all the groups tested. Of note, nicotine treatment alone avoided, in some level, the rotenone-induced p-TH reduction within the OB in males. In this sense, an even more pronounced p-TH increment is observed for both; nicotine b.s. and nicotine + caffeine treatments in females rotenone-exposed rats. This result is

also supported by the lack of TH expression decrease in the rotenone groups in a scenario of TH upregulation for these very groups in the sham conditions. It is reported that several caffeine (10mg/kg i.p.) or even selective A2a antagonists like SCH58261 (2 mg/kg i.p.) administrations are associated with elevated striatal DA concentrations and increased TH phosphorylation in mice (Hsu et al., 2010). Other studies showed nicotine-induced TH phosphorylation in cervine (Knowles et al., 2011) and bovine (Bobrovskaya et al., 2007), leading to activated and sustained catecholamine biosynthesis. Probably, nicotine triggered a similar effect in the OB of male and female rats. However, such pro-phosphorylation mechanism induced by caffeine and nicotine have a mild impact in the olfactory discrimination, especially in females.

Regarding olfactory disorders in PD, no clinical study has tested caffeine or similar drugs for such purpose. In fact, acute caffeine administration (10mg/kg) proved to be effective in reversing age-related olfactory discrimination deficit in 12-month-old rats (Rui D.S. Prediger et al., 2005). Also, women are less affected by olfactory loss than men, and the same occurs to PD olfactory disturbances (R. L. Doty, Stern, Pfeiffer, Gollomp, & Hurtig, 1992; R. Liu et al., 2015). In fact, in our rotenone model, female rats did not present olfactory discrimination impairment in the ODT, consequently, a higher ODI was obtained for all the groups. It is noteworthy that such outcome could be due to some level of influence of the ovarian hormones, positively impacting the olfactory performance (Richard L. Doty, Applebaum, Zusho, & Settle, 1985; Richard L. Doty & Cameron, 2009). Another point that should be mentioned is the possible cognitive influence over the olfactory task. In view of that, we executed a SRT to access the social memory, which is often impaired in different PD models (Rui D S Prediger et al., 2010) (Aguiar Jr et al., 2009; R. D S Prediger, Cunha, & Takahashi,

2005). However, intranigral rotenone administration was not able to produce such deficit in male rats, although, with some impairment in sham nicotine (male), rotenone caffeine + nicotine (male) and rotenone saline (female) groups.

In agreement with our hypothesis suggesting the activation of dopaminergic periglomerular neurons as a determinant factor for olfaction, we observed that ODI impaired groups are associated with two different/opposite conditions: (a) elevated OB TH phosphorylated protein density (Fig. 13A-B) + elevated SNpc TH-ir neuronal density (Fig. 13B-C); and (b) low OB TH phosphorylated protein density (Fig. 13A-B) + low SNpc TH-ir neuronal density (Fig. 13B-C). In addition, all the three best performance ODI groups are represented in the middle of those quantifications diagrams (Fig. 13B;D), indicating a balance of these two factors for the olfactory process don't be damaged.

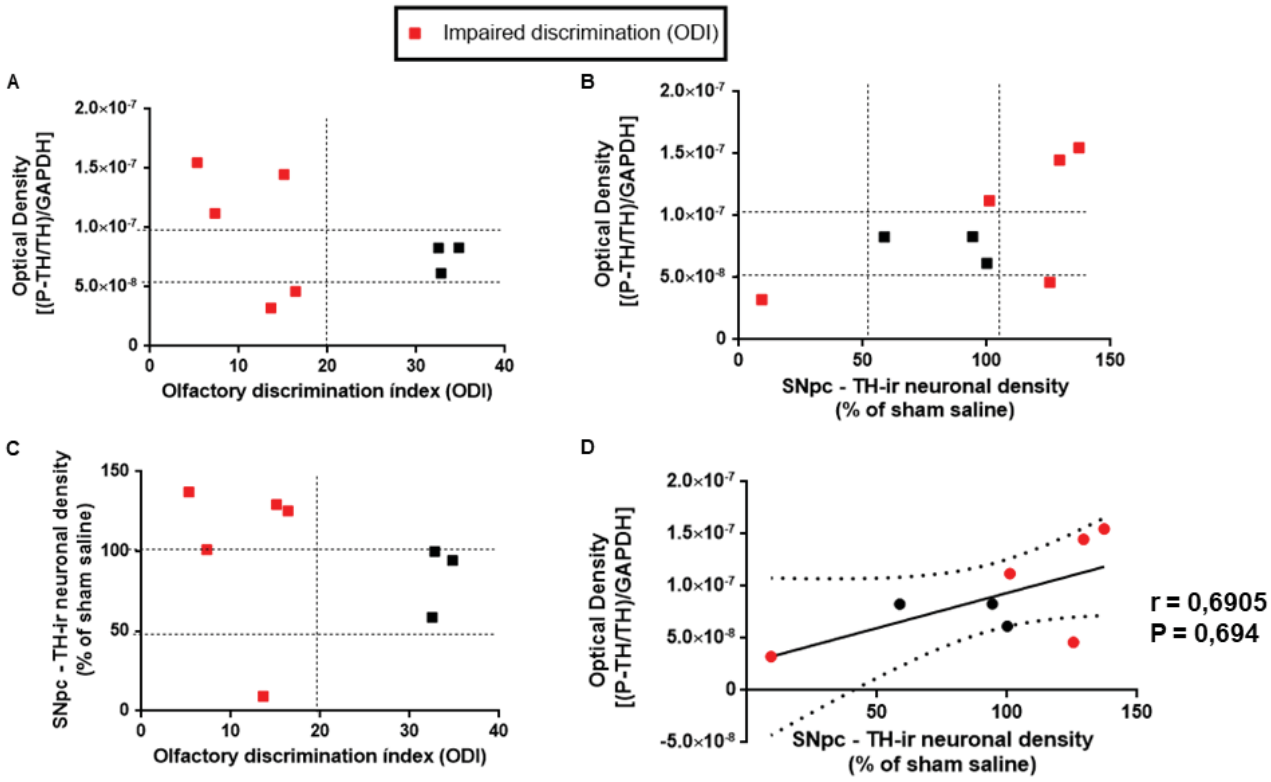

Figure 13. Male groups distribution diagrams**.** Group's mean are used to represent the groups distribution, based on X/Y factors. Red color = impaired olfactory discrimination index groups. Black color = olfactory discrimination index bests performances groups. **D.** Spearman's correlation among SNpc TH-ir neuronal density and OB p-TH protein expression for all mean groups. R=coefficient related to a moderate positive correlation.

Accordingly, the OB olfactory process is D2 inhibitory receptors activation-dependent, present in the olfactory receptor neurons in the OB GL (Richard L. Doty, 2012b; Richard L. Doty & Risser, 1989; Gutièrrez-Mecinas et al., 2005; Koster et al., 1999; O'Connor & Jacob, 2008). The hyperactivation of these receptors (in a supposed DA excess condition) leads to a blocked glutamate release, and impaired subsequent activation of AMPA and NMDA receptors present in mitral and tufted cells, causing hyposmia or anosmia. On the other hand, the hypoactivation of OB D2

receptors (in a supposed DA limited condition) lead to a glutamate release by the olfactory receptor neurons, which going to activate the AMPA and NMDA receptors in mitral and tufted cells, but going to activate also, the DA/GABA periglomerular neurons, increasing the dopamine release and facilitating GABA responses. GABA could block the mitral and tufted cells, leading to the same point: hyposmia or anosmia. Accordingly, a balance among this activation/deactivation DA-dependent olfactory synapses is the best condition to the olfactory discrimination normally occur. Interesting, we did not observe the same pattern of groups distribution comparing the TH non-phosphorylated form expression in the OB or the OB TH-ir neuronal counting (Fig. 14A-C). However, there is a “lesion distribution” for TH non-phosphorylated expression in the OB (Fig. 14D).

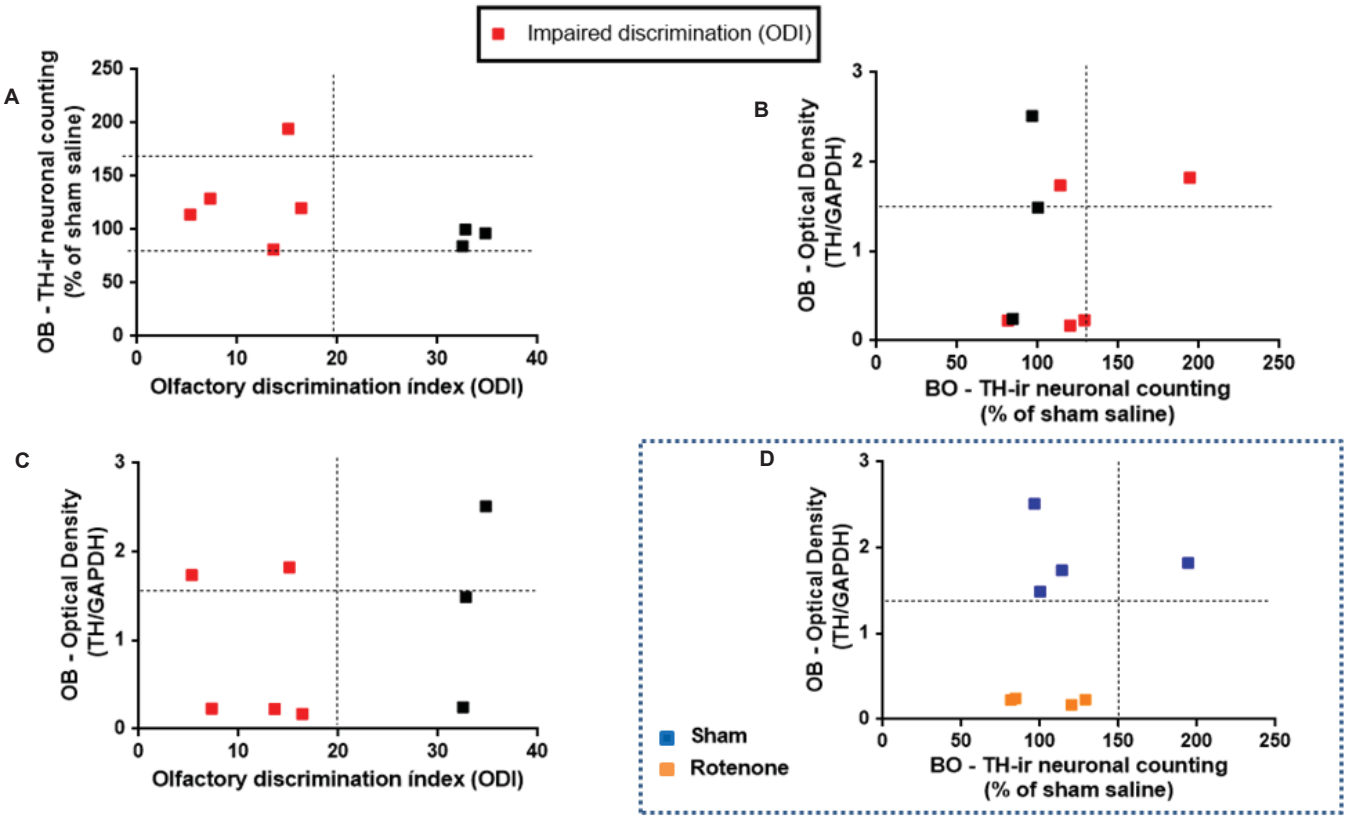

Figure 14. Male groups distribution diagrams**.** Group’s mean are used to represent the groups distribution, based on X/Y factors. Red color = impaired olfactory discrimination index groups. Black color = olfactory discrimination index bests

performances groups. **D.** Lesion distribution groups among BO TH-ir neuronal counting and OB TH-ir protein expression for all mean groups. Blue color = sham groups and orange color= rotenone groups.

Apparently, the OB TH-phosphorylated expression and the SNpc TH-ir neuronal density are directly related to the olfactory function, in males, supporting our hypothesis. Otherwise, females did not present similar patterns to males (Figure 15A-G), probably caused by an absent olfactory disturbance, even in the presence of a prominent dopaminergic lesion caused by rotenone. Therefore, all the groups exhibited a high performance in the ODI, independent of decreased OB TH-phosphorylation expression (for most of groups) or increased SNpc TH-ir neuronal density, counteracting males. At the same time, a “lesion distribution” of groups was also present in females (Fig. 15G), related to the SNpc TH-ir neuronal density, but not influenced by ODI.

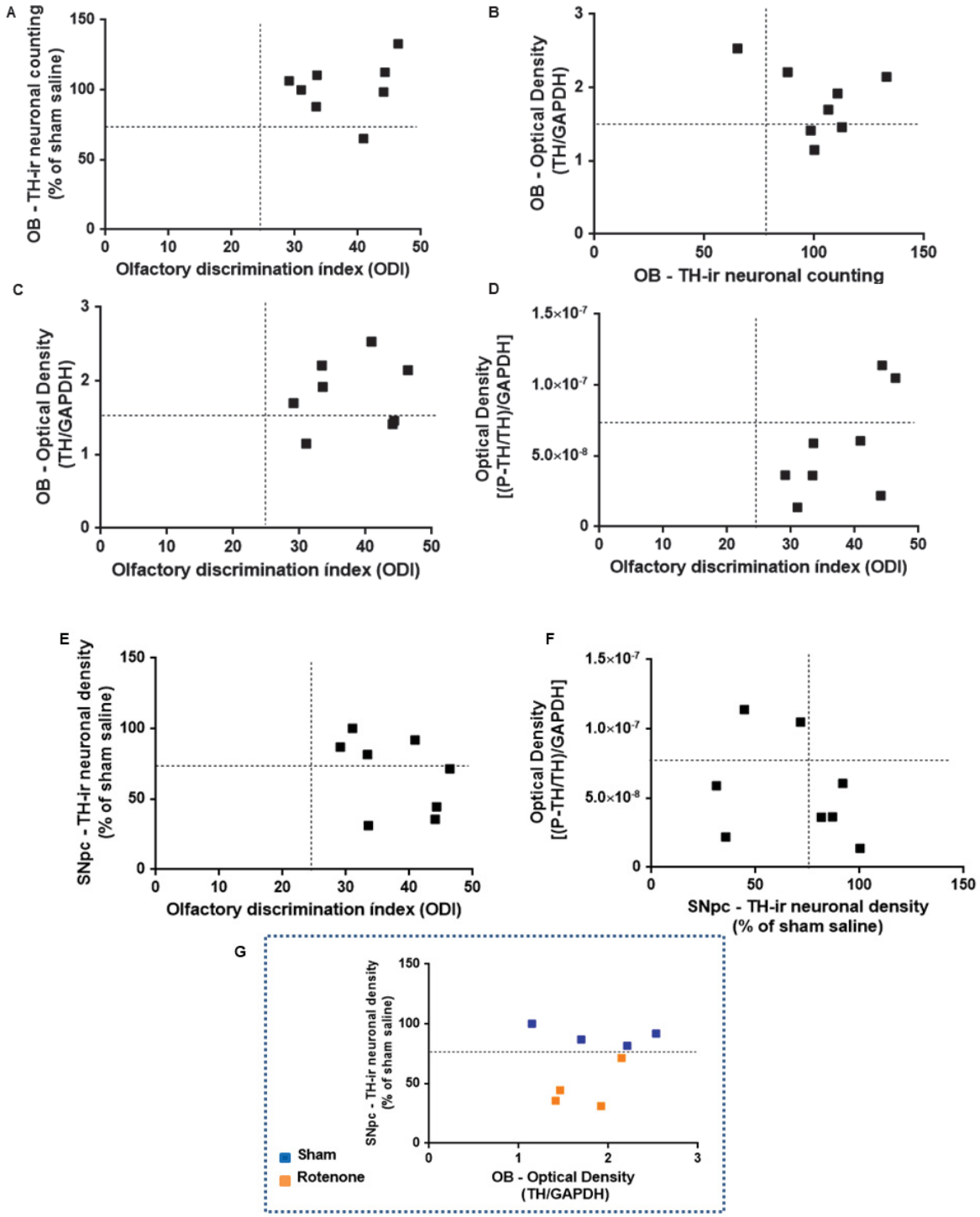

Figure 15. Female groups distribution diagrams**.** Mean groups are used to represent the groups distribution, based on X/Y factors. Black color = olfactory discrimination index bests performances groups. **G.** Lesion distribution groups among BO TH-ir

neuronal counting and SNpc TH-ir expression for all mean groups. Blue color = sham groups and orange color = rotenone groups.

In conclusion, caffeine, but not nicotine is able to produce relevant improvements in olfaction in the intranigral rotenone model of PD. Besides, it is proposed that the mechanism behind this effect is associated to the TH enzyme phosphorylation. On the other hand, nicotine also increases TH phosphorylation, but in an exacerbated level that impairs olfactory function. Partial restoration of dopaminergic neurons in the SNpc, for both caffeine and nicotine treatments, provide new neuroprotective evidence in the rotenone PD animal model. Additionally, the results suggest that there are sex-differences in the rotenone PD animal model, which similarly occur in a human population, meaning that are necessary sex-differentiated therapies.

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Conflicts of Interest

There are no conflicts of interest.

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4 ARTIGO CIENTÍFICO NUMERO 2

Parkinson's disease: modulating A2a receptors on the odor hand.

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Abstract

Parkinson's disease (PD) is characterized by the loss of dopaminergic neurons of the substantia nigra pars compacta (SNpc), leading to a dopamine reduction in the striatum and, consequently, to motor impairment related to the disease. However non-motor symptoms are also present in the PD pre-motor phase, such as olfactory disorder, observed in up to 90% of patients. Studies have shown that there is an inverse association between caffeine consumption and the risk of developing PD, suggesting A2a antagonist drugs may have a neuroprotective effect. The aim of this study was to investigate the effects of acute A2a agonist and antagonist drugs olfactory bulb (OB) administrated on the olfactory discrimination of rats submitted to intranigral rotenone injury. Male and female Wistar rats (90 days) received CGS 21680 (A2a agonist) or SCH 58261 (A2a antagonist) microinfusions in the OB mitral cell layer seven days after SNpc rotenone lesion. After that, animals performed the olfactory discrimination task, and then the OBs were dissected for western blot analysis or perfused for TH immunohistochemistry. The results demonstrated that SCH 58261 restores the olfactory discrimination impaired by rotenone injury in males, but no effect was present in female rotenone group. On the opposite, CGS 21680 caused olfactory discrimination impairment, similar to rotenone, in both male and female. The OB TH-ir neuronal counting indicates an increased cell number in the male rotenone SCH58261 and female sham SCH 58261 groups, which is related to the better olfactory discrimination index scores. Neither the CGS 21680 nor the SCH 58261 were able to increase OB TH phosphorylation in males or females, indicating low levels of TH neuronal activity and dopamine release. In summary, the A2a antagonist drug was effective in improving the olfactory function impaired in the male PD animal model, possibly by interaction with the dopaminergic system, but the mechanisms are still not understood.

Key-words: Parkinson’s disease; olfactory bulb; tyrosine hydroxylase; A2a receptors; rotenone; sex-dymorphism.

Introduction

Parkinson's disease (PD) is associated with dopaminergic system disturbance (Lang & Lozano, 1998; Lima, 2012), and conquently this neurotransmission system is the prototypical target for the L-3,4-dihydroxyphenylalanine (L-DOPA) therapy for the motor symptoms. However, L-DOPA is not effective for the numerous non-motor disturbances, such as olfactory disorders (Richard L. Doty, 2017), which affect about 90% of PD patients, beginning years before the motor onset (Braak et al., 2003a; R L Doty et al., 1988; G. Webster Ross et al., 2008). Therefore, the search for new therapies that can improve the olfactory function and, above all, prevent or reverse neurodegeneration is needed.

The adenosinergic system has been investigated for its benefits in relation to neuroprotection associated with neurodegenerative diseases, such as PD (Franco & Navarro, 2018). In view of that, there is an inverse relationship between caffeine (a non-selective antagonist of adenosinergic receptors) consumption and the onset of PD (Ascherio et al., 2001). In addition, it is known that caffeine and a selective A2a receptor antagonist (SCH58261) have shown a neuroprotective effect against the 1-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP)-induced dopamine (DA) depletion in the striatum (Kui Xu et al., 2002). Besides, another possible mechanism of caffeine is phosphodiesterases inhibition, which could increase the phosphorylation and, consequent activity, of the tyrosine hydroxylase (TH) enzyme (Bobrovskaya et al., 2007), which is the rate-limitating step in the DA biosynthesis (Molinoff & Axelrod, 1971). Moreover, both chronic caffeine and the selective A2a antagonist (SCH 58261) were able to enhance TH phosphorilation in the striatum, thus promoting locomotor sensitization (Hsu et al., 2010).

Based on those evidence, the aim of this study was to investigate whether an acute pharmacological modulation of the A2a receptors, present in the olfactory bulb (BO), of male and female rats would be able to restore the olfactory function after an intranigral lesion produced by the neurotoxin rotenone.

Material and Methods

Ethics statement

The experiments were carried out according to the guidelines of Brazilian Guide for Care and Use of Laboratory Animals (COBEA) and were approved by the Federal University of Paraná (UFPR) Ethics Committee (approval ID #852).

Animals

Male and female Wistar rats from UFPR bred colony, weighing 250-280 g (approximately 3 months old) were used. The animals were randomly housed in groups of five in polypropylene cages with sawdust and maintained in 22±2°C on a 12:12-h light-dark cycle (lights on at 7:00 AM) with free access to water and food, except during the behavioral test.

Experimental design

Before surgeries, the animals were distributed randomly in two groups: sham (n=45 – male; n=45 - female) and rotenone (n=45 – male; n=45 - female). After the surgery procedure, they were redistributed in six groups (n=15/group), considering the pharmacological modulation by CGS21680 (a selective A2a agonist) and SCH58261 (a selective A2a antagonist), as follows: sham vehicle, sham CGS21680, sham SCH58261, rotenone vehicle, rotenone CGS58261, rotenone SCH58261. On day 0

(Figure 1) a stereotaxic surgeries were performed for substantia nigra pars compacta (SNpc) rotenone lesion or vehicle infusion (dimethylsulfoxide (DMSO) - to sham groups). On day 5, the animals were isolated for the sawdust collection required for the olfactory discrimination task (ODT). On day 7, twenty minutes after the drug infusion by the OB cannulas, the animals were submitted to the behavioral tests and, immediately after, their brains were dissected or perfused for further analysis.

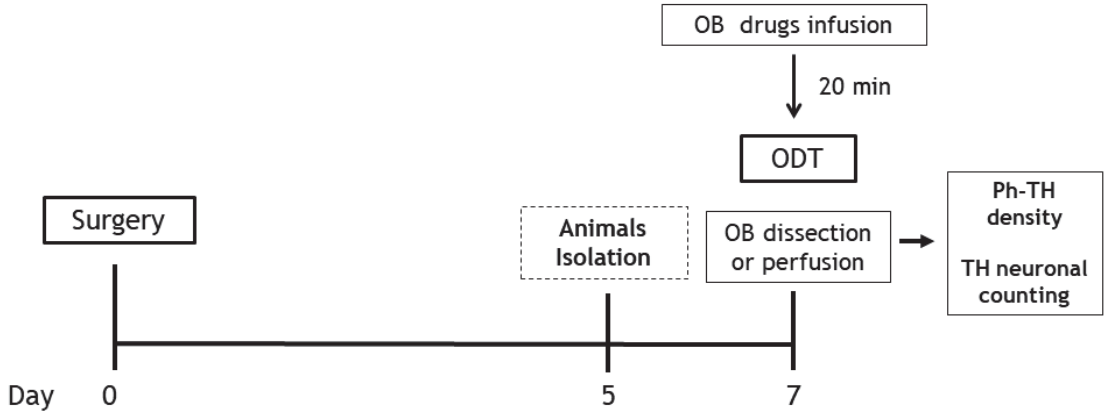

Figure 1. Schematic representation of the experimental design. OB: olfactory bulb; ODT: olfactory discrimination task; TH: tyrosine hydroxylase.

Stereotaxic Surgery

The animals were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). The following coordinates were used to the bilateral injury, with bregma as a reference: SNpc (AP) = - 5.0 mm, (ML) = ± 2.1 mm e (DV) = - 8.0 mm (Paxinos & Watson, 2005). Needles were guided to the region of interest for a bilateral infusion of 1 µL of rotenone (12 µg/µL) (Sigma-Aldrich, United States) or of DMSO (Sigma-Aldrich, United States) using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0,33 µL/min for 3 minutes (AURICH et al., 2017; NOSEDA et al., 2014; RODRIGUES et al., 2014; TARGA et al., 2018).

Complementarily, bilateral guide cannulas were implanted in the OB of each rat allowing a posterior 1 µL infusion of CGS21680 (2 nmol/µL) (Tocris Bioscience®, Bristol, UK), SCH58261 (56 nmol/µL) (Tocris Bioscience®, Bristol, UK) or vehicle (DMSO) at a rate of 0,33 µL/min for 3 minutes, in their respective groups. Coordinates with reference to bregma for implantation of guide cannulas were: (AP) = +7.80 mm (ML) = ±1.5 mm and (DV) = -1.0 mm (Paxinos & Watson, 2005). On day 7, all animals received the last OB administration 20 minutes before the behavioral tests. A2a selective drugs doses were chosen according to the previous literature report (Wang et al., 2017).

Olfactory discrimination task

The version was modified from (Rui D.S. Prediger et al., 2006b) and previously reported (Ilkiw et al., 2018; Rodrigues et al., 2014). Briefly, the apparatus consists in a box (60 x 40 x 50cm), equally divided into two compartments connected by an open door. Before the test, it was performed an adaptation period to the apparatus of 2 minutes, in which both compartments was with fresh sawdust. After that, clean sawdust is added on one side of the box (non-familiar odor). On the other side of the box, is added sawdust which animals remained isolated for 48 hours before testing (familiar odor). Each animal was placed in the middle of apparatus and video recorded up to 3 min, to evaluate the investigation time of each compartment. The animal that shows olfactory discrimination impairment explores both compartments equally, indicating absence of discrimination. As a measure of discrimination, an “olfactory discrimination index (ODI)” was calculated by dividing the difference in exploration time between the two compartments (compartment non-familiar - compartment familiar) by the total amount of exploration for both compartments

(compartment non-familiar + compartment familiar). ODI was then multiplied by 100 to express it as a percentage of de mean.

TH- immunohistochemistry within the OB

Animals were deeply anesthetized with ketamine immediately after the behavioral tests and were intracardially perfused with saline, then with 4% of the formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and kept for 48 h immersed in fixative solution at 4ºC. The samples were immersed in 30% sucrose solution for 3 days and finally frozen at -80ºC. Sections (40 µm) were obtained from the OB (+7.80 mm and +7.08 mm) (Paxinos & Watson, 2005; Rodrigues et al., 2014). The sections were incubated with primary mouse anti-TH antibody (1:8000 - OB; Sigma Aldrich, Missouri, USA - #T2928) prepared in phosphate-buffered saline containing 0.3% Triton X-100 overnight at 4°C. Biotin-conjugated secondary antibody incubation (1:200 anti-mouse #Vector Laboratories, USA), was performed for 2h at room temperature. After several washes in phosphate-buffered saline, antibody complex was localized using the ABC system (Vectastain ABC Elite kit, Vector Laboratories, USA) followed by 3,3´- diaminobenzidine reaction with nickel enhancement. Cell counts for the glomerular layer (GL) of the OB were carried out by the software Image-Pro Express 6. The mean number of TH-immunorreactive (TH-ir) neurons in each hemisphere was considered to be representative of the OB neuronal cells in each animal. For each group, a mean value was calculated (percentage relative to the sham control), and compared with those of the other groups. The images were obtained through the use of a motorized Axio Imager Z2 microscope (Carl Zeiss, Jena, DE), equipped with an automated scanning VSlide (Metasystems, Altlussheim, DE).

Western blot analysis

To determine TH and the TH phosphorylated form (p-TH) within the OB, after the ODT, animals were decapitated and brains were rapidly ice-removed and OBs were dissected. Until processed for analysis, tissues were stored at -80ºC. Samples were sonicated in lysis buffer containing 150mM NaCl, 50mM Tris-HCl, 2mM EDTA, 1% Triton X-100, 1mM PMSF, 1mM sodium orthovanadate (Na3VO4), 1mM sodium fluoride (NaF) and complete protease and phosphatase inhibitor mixture EDTA-free (Roche). After centrifugation (10 min, 12,000xg, at 4°C), supernatant was collected and protein concentration was determined by the Bradford method (Bio-Rad, Germany). Samples were subjected to SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) and proteins transferred to a nitrocellulose membrane (GE Healthcare). The membranes were then blocked in 2% BSA diluted in TBS-T (blocking solution) for 1h at room temperature and incubated overnight with the desired antibody diluted in blocking solution. The antibodies used were: mouse monoclonal anti-GAPDH (code SC-32233, Santa Cruz), anti-Tyrosine hydroxylase (TH) (code B2409, Santa Cruz), anti-phosphorylated-Tyrosine Hydroxylase (p-TH) ((Ser40) code 2791, Cell Signaling). After primary antibody incubation, membranes were extensively washed with TBS-T and incubated with HRP-conjugated secondary antibody (Sigma) in blocking solution for 1h at room temperature. After p-TH incubation, membranes were stripped and immunobloted against TH. Finally, membranes were washed again and immune complexes were detected using the ECL chemiluminescent detection system (GE Healthcare Life Sciences, Brazil). The protein levels were quantified by densitometry using ImageJ v1.47 software (National Institutes of Health, USA).

Statistical analysis

The ODT was analysed by three-way repeated measures ANOVA (factors: lesion; treatment and compartment for ODT) followed by Bonferroni´s post hoc test. The olfactory discrimination index (ODI), TH-immunohistochemistry and Western blot quantifications were analyzed by two-way ANOVA (factors: lesion and treatment; or lesion and sex differences for male X female comparisons) followed by Bonferroni´s post hoc test. One-way ANOVA followed by Bonferroni´s post hoc test was used to compare the western blot analysis among males and females with the same lesion factor. Values were expressed as mean (ODI) or mean ± standard error of the mean (SEM). Significant differences were set at $P \leq 0.05$. For groups distribution representative visualization, mean values were plotted in a scatter diagram (Cartesian coordinates), comparing behavioral, molecular and histochemical parameters.

Results

Olfactory discrimination task (ODT) and olfactory discrimination index (ODI)

The ODT performed by male rats reveled that sham vehicle ($P<0.01$) and sham SCH 58261 ($P<0.05$) groups had a significant olfactory discrimination, spending more time in the familiar odor compartment (Figure 2A). A similar result was observed in the rotenone SCH 58261 group ($P<0.001$), indicating an improved discriminatory ability, even when the lesion was present. Analyzing the male ODI (Figure 2B), no differences were found among groups, with influence of treatment effect [$F(2.50)=3.91$; $P=0.02$], but without significant lesion effect [$F(1.50)=0.3235$; $P=0.5720$] and interaction [$F(2.50)= 0.68$; $P=0.51$].

Female rats presented no significant differences either in the ODT (Figure 2C), except by the sham SCH 58251 group (P<0,01), that spent more time in the familiar compartment. Similar results were found in the female ODI, indicating that sham SCH 58261 group had the best discrimination performance and sham CGS21680 group had the worse index (Figure 2D).

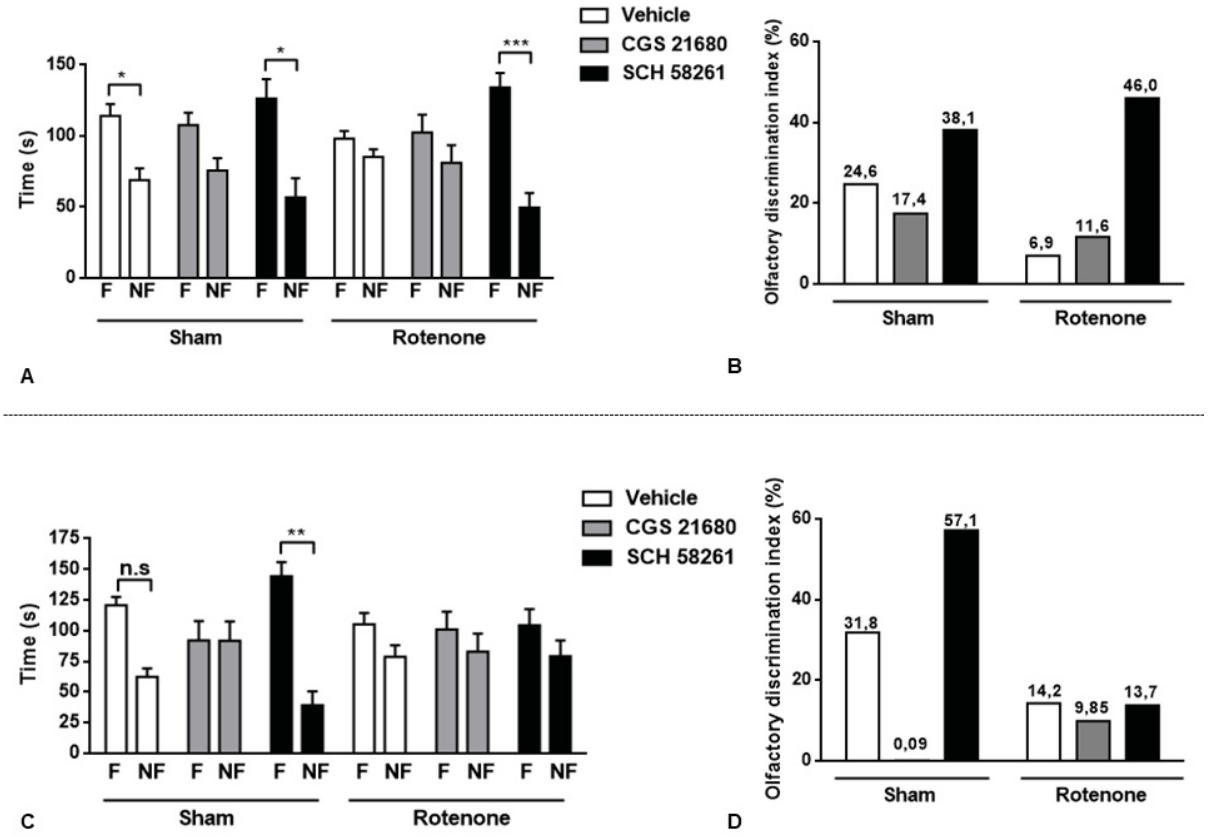

Figure 2. Olfactory discrimination task (ODT) and olfactory discrimination index (ODI). Time (s) spent in familiar (F) and non-familiar (NF) odor compartments in the ODT 7 days after surgery. ODI was calculated by (NF-F/NF+F)*100. **A**. Male groups olfactory performance in the ODT. **B**. Males ODI means by group. **C**. Female groups olfactory performance in the ODT. **D**. Female ODI means by group. The bars are represented as mean ± standard error of the mean (ODT) or as group's mean (ODI). N=6-10/group, *P≤0.05, **P≤0.01, ***P≤0.001. Three-way ANOVA followed by

Bonferroni post hoc test (ODT) and Two-way ANOVA followed by Bonferroni post hoc test (ODI).

TH-immunohistochemistry within the OB

The OB TH-ir neuronal counting was higher in the rotenone SCH 58261 group compared to rotenone vehicle ($P<0.05$), associated with a significant treatment effect [$F(2.16)=7.93$; $P=0.004$] (Figure 3A-B). Lesion effect [$F(1.16)=0.49$; $P=0.49$] and interaction [$F(2.16)=1.94$; $P=0.17$] had no influence.

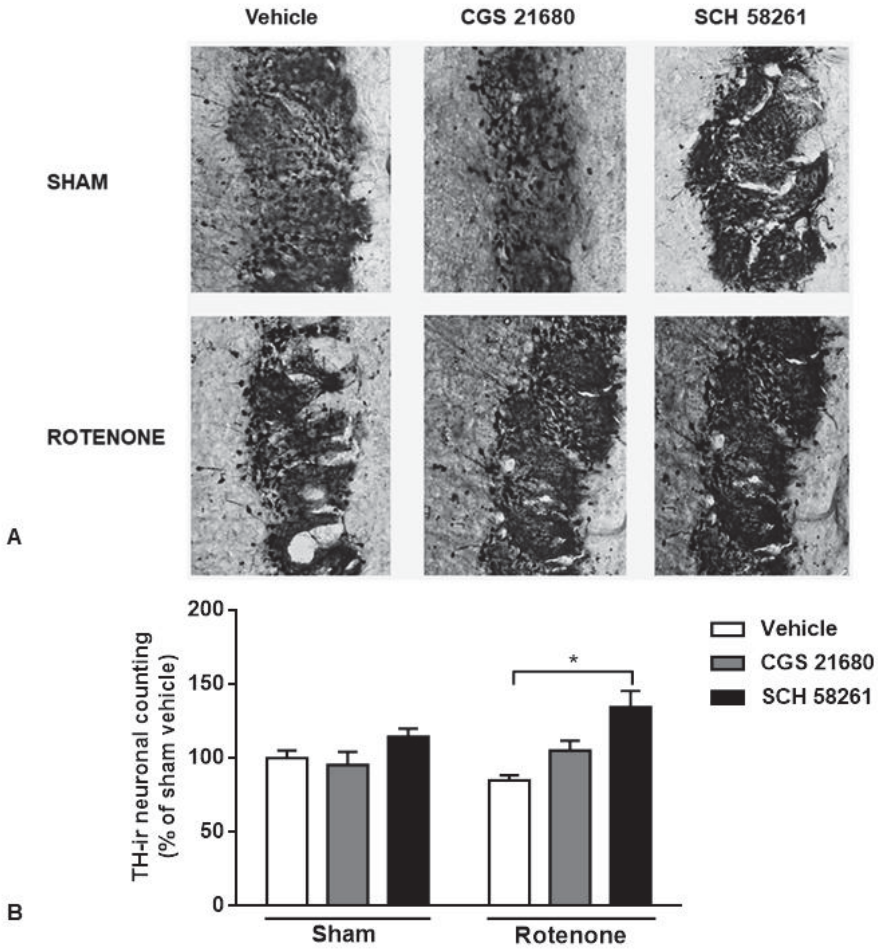

Figure 3. Percentage of TH-ir neurons in the glomerular layer within the OB of male rats. **A.** Representative images of dopaminergic neurons within the OB (magnification 50x). **B.** Corresponds to OB neuronal counting analysis. The bars are represented as

mean ± standard error of the mean, N=2-3/group, *P≤0.05. Two-way ANOVA followed by Bonferroni post hoc test.

Analyzing the females OB GL (Figure 4A-B), the TH-ir number was increased in the sham SCH 58261 group, compared to sham CGS21680 (P<0.01) and to rotenone SCH 58261 (P<0.001) groups, with lesion [F(1.18)=12.83; P=0.0021] and treatment [F(2.18)=5.052; P=0.0181] effects and also with interaction [F(2.18)=10.68; P=0.0009].

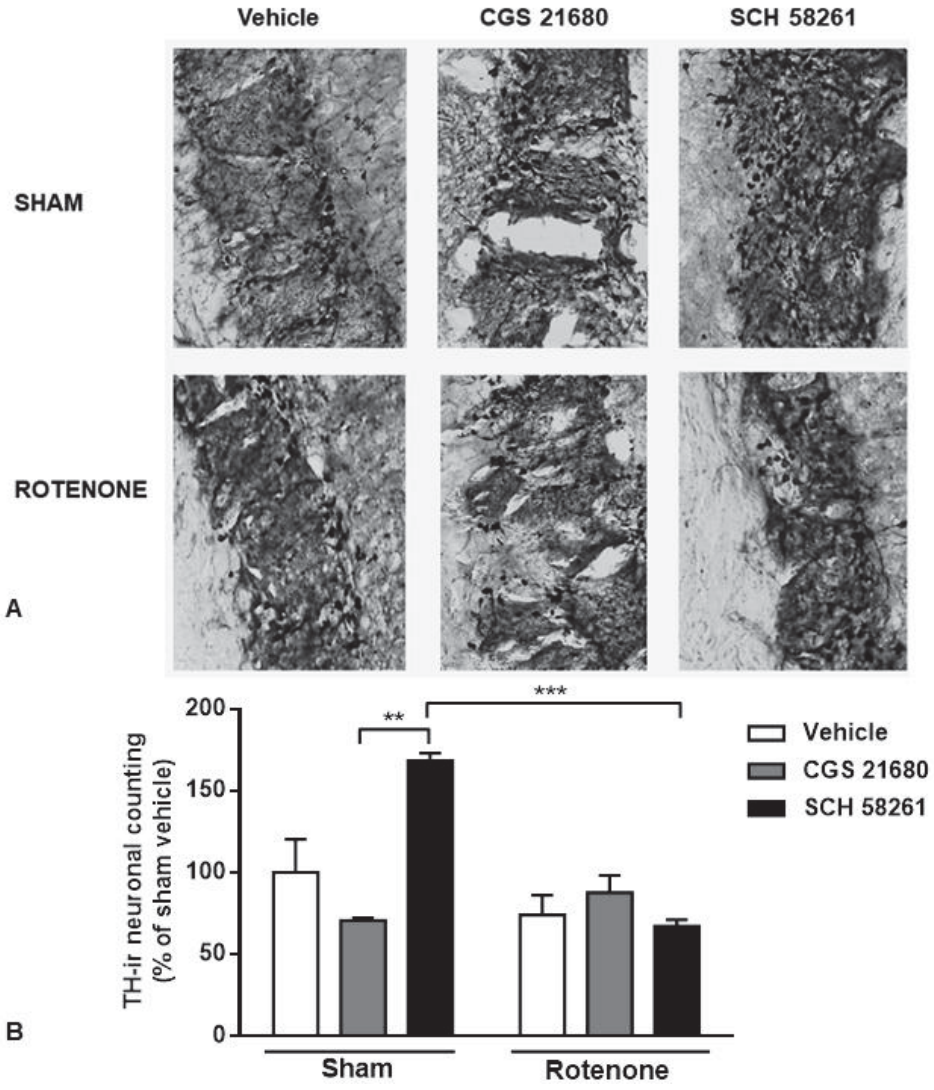

Figure 4. Percentage of TH-ir neurons in glomerular layer within the OB of female rats. **A.** Representative images of dopaminergic neurons within the OB (magnification

50x). **B.** Corresponds to OB neuronal counting analysis. The bars are represented as mean ± standard error of the mean, N=3/group. **P≤0.01, ***P≤0.001. Two-way ANOVA followed by Bonferroni post hoc test.

Comparing male and female (Figure 5 A-C), only SCH 58261 OB administration demonstrated an increased GL TH-ir cells number in the female sham group compared to male sham group (P<0.01), with significant treatment effect [F(1.12)=29.64; P=0.0001] (Figure 5C). On the opposite, male rotenone group presented an increased TH-ir neurons number compared to rotenone female group (P<0.001), but without significant sex difference effect [F(1.12)=0.76; P=0.39]. Besides that, a significant interaction was found [F(1.12)=65.93; P<0.0001].

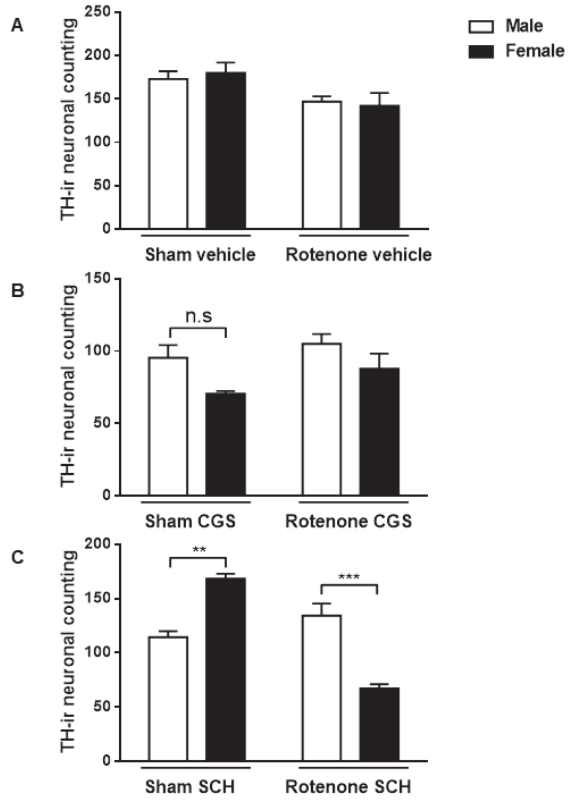

Figure 5. Comparisons among male and female groups concerning OB neuronal counts. **A.** Vehicle; **B.** CGS21680 (A2a agonist); **C.** SCH58261 (A2a antagonist). The bars are represented as mean ± standard error of the mean. N=3/group, **P≤0.01,

***P≤0.01. Two-way ANOVA followed by Bonferroni post hoc test. N.s.: non-significant difference.

Western blot OB analysis

Male (Figure 6) and female (Figure 7) OB phosphorylated-TH (p-TH) densities were measured, but no significant differences were found, even segregating data by lesion factor (Figure 6CD and Figure 7CD).

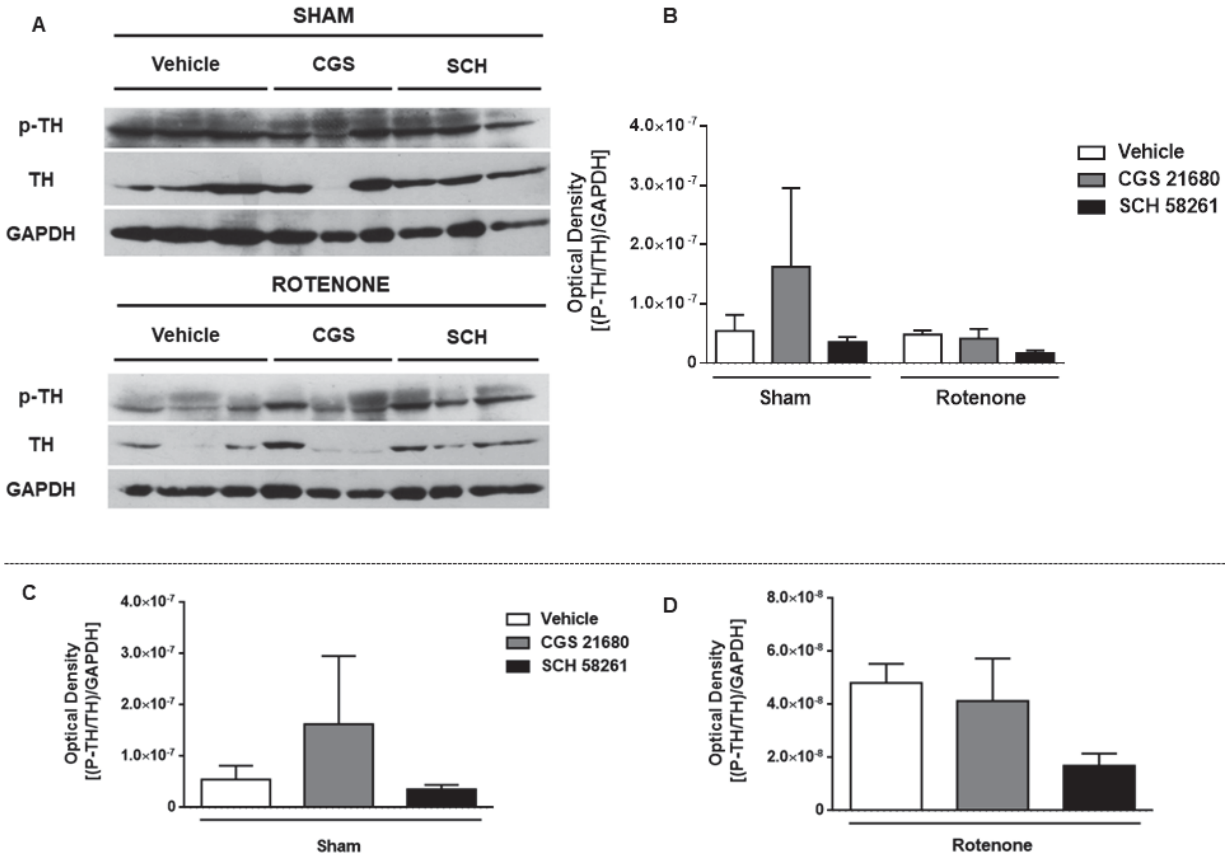

Figure 6. Western blot analysis of the normalized p-TH expression within the OB of male rats. **A.** Representative images of the bands from male groups; **B.** Optical densities comparison of normalized p-TH expressions; **C.** Analysis of males OB p-TH densities of sham groups; **D.** Analysis of males OB ph-TH densities of rotenone

groups. The bars are represented as mean ± standard error of the mean, N=3/group. Two-way ANOVA followed by Bonferroni post hoc test. p-TH: tyrosine hydroxylases protein phosphorylated form; TH: tyrosine hydroxylase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

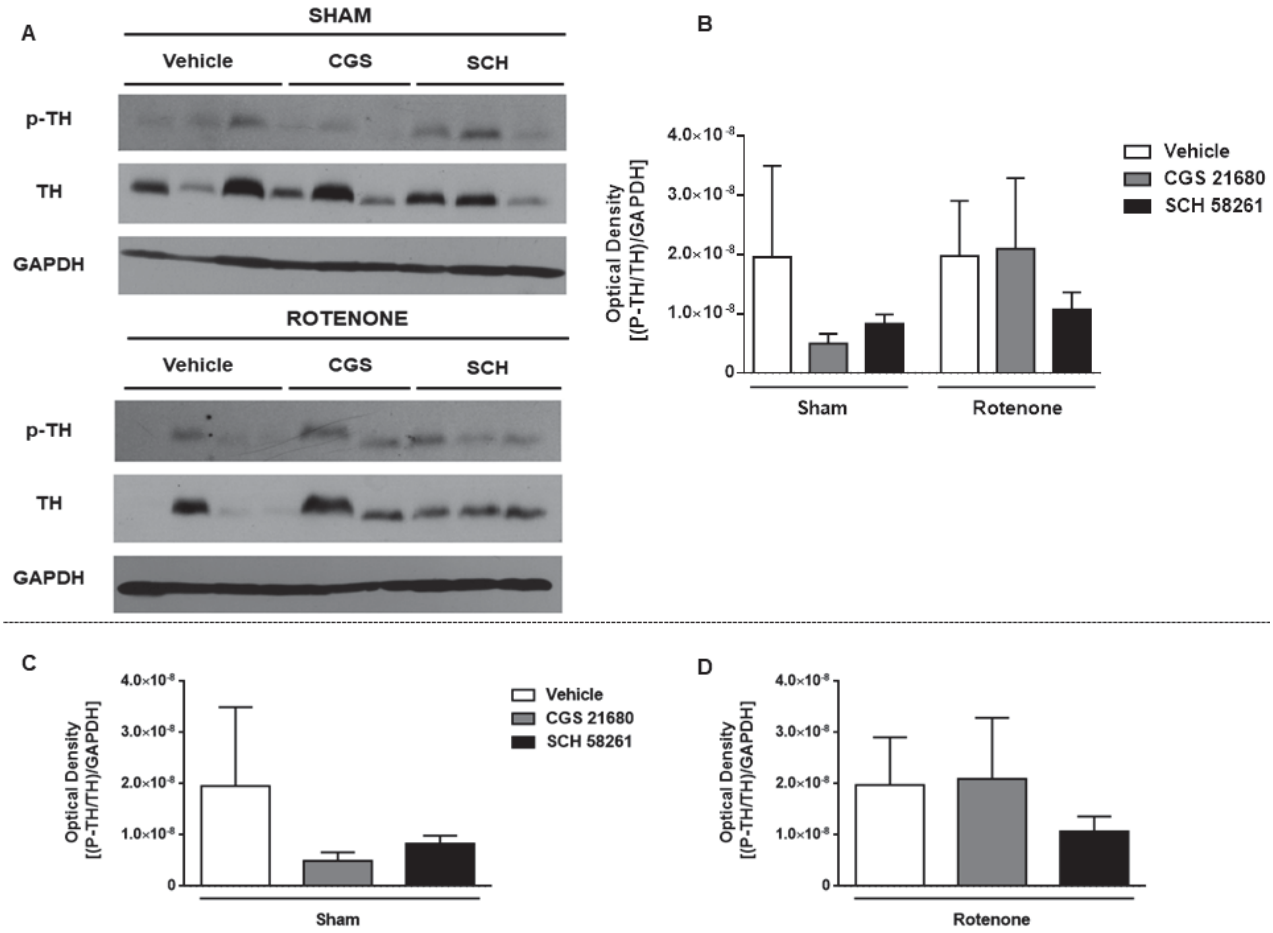

Figure 7. Western blot analysis of the normalized p-TH expression within the OB of female rats. **A.** Representative images of the bands from female groups; **B.** Optical densities comparision of normalized p-TH expression; **C.** Analysis of females OB p-TH densities of sham groups; **D.** Analysis of females OB p-TH densities of rotenone groups. The bars are represented as mean ± standard error of the mean, N=3/group. Two-way ANOVA followed by Bonferroni post hoc test. p-TH: tyrosine hydroxylases

protein phosphorylated form; TH: tyrosine hydroxylases; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Comparing male and female OB p-TH protein densities by treatments (Figure 8A-C), only SCH 58261 OB administration produced a higher density in sham male group compared to sham female group ($P<0.05$), with significant sex difference effect [$F(1.8)=10.38$; $P=0.0122$], but no treatment effect was present [$F(1.8)=2.464$; $P=0.1551$]. However, a significant interaction was noted [$F(1.8)=4.134$; $P=0.0765$].

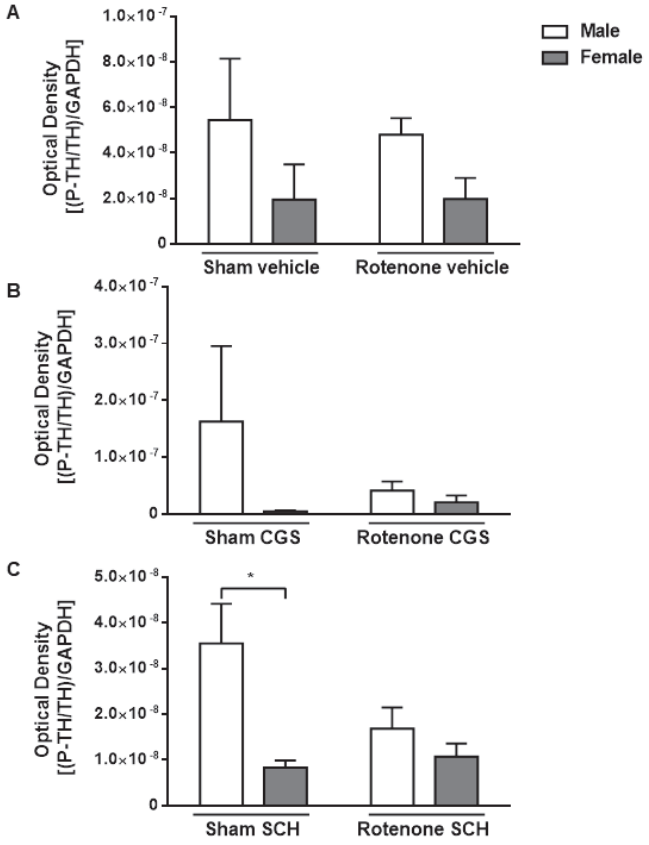

Figure 8. Comparison among male and female groups concerning normalized p-TH expression within the OB. **A.** Vehicle; **B.** CGS21680; **C.** SCH58261. The bars are represented as mean ± standard error of the mean. N=3/group, *P≤0.05. Two-way ANOVA followed by Bonferroni post hoc test.

Discussion

According to our previous study (not published), male Wistar rats that received intraperitoneal caffeine injections (a non-selective A2a antagonist) presented an olfactory discrimination improvement, after rotenone-induced olfactory impairment. This is associated to the increased levels of TH phosphorylation within the OB, producing olfaction restoration. Also, female rats appear to be more resistant in terms of olfactory performance, after the nigrostriatal lesion, even in the context of increased TH phosphorylation. In this sense, the present study show similar results of olfactory improvement in male rats due to a selective A2a antagonist (SCH58261) detected by means of the ODT. Otherwise, a selective A2a agonist (CGS21680) impaired the olfactory performance recorded by the ODT in both sexes, suggesting that A2a receptor is a key player in the olfactory processing. Indeed, A2a receptors are highly expressed in the OB (KAELIN-LANG; LAUTERBURG; BURGUNDER, 1999; RIBEIRO; SEBASTIÃO; DE MENDONÇA, 2002; ROTERMUND et al., 2018; SEBASTIÃO; RIBEIRO, 1996) and its blockade is associated with an olfactory improvement in aged and middle-aged rats (Rui D.S. Prediger et al., 2005). Complementarily, that study also discarded the participation of A1 receptors in mediating olfactory responses due to the absence of olfaction changes after an A1 antagonism.

The mechanism underlying this process is still far from being fully understood, but there are some evidence, although not in the OB, suggesting that the adenosinergic system interacts with the dopaminergic system in the striatum, thorugh heteromers formation (Sergi Ferré et al., 2007). Accordingly A2a agonists inhibits the D2 receptors activation effects, and an A2a antagonist potentiates the D2 receptors activation effects (S Ferré, O'Connor, Fuxe, & Ungerstedt, 1993). However, a binding

study suggested that adenosine receptors are present in OB periglomerular neurons, while D2 receptors are located within the odor receptor neurons (Johansson, Georgiev, & Fredholm, 1997), thus, not allowing the heteromers formation. Therefore, other mechanisms not described are possibly involved in these two systems signaling in the OB.

Regarding the OB TH-ir neuronal counting, we observed a relation between higher ODI performances and increases in the number of TH-ir neurons in the OB, for both male (Figure 9C) and female (Figure 10C) with SCH 58261 OB infusion. The acute A2a antagonist infusion produced an apparent increase in TH-ir GL expression, not demonstrated in the present study (Figures 9B and 10B) probably because the TH expression was analyzed in the entire OB structure, while neuronal counting were made only in the OB GL. Increased SNpc TH mRNA expression is observed in rats with chronic caffeine administration (9 days) (Datta et al., 1996) and a non-significant increase in TH striatal expression is reported for chronic caffeine and SCH 58261 administrations in mice (Hsu et al., 2010), similarly to our findings in male rats (data not shown). Besides, the sex comparison revealed an increment of TH-ir neuronal counting associated to sham female SCH58261-treated, but not for the equivalent rotenone group. Also, such effect of SCH58261 treatment was related with higher ODI scores obtained from female sham and rotenone male groups.

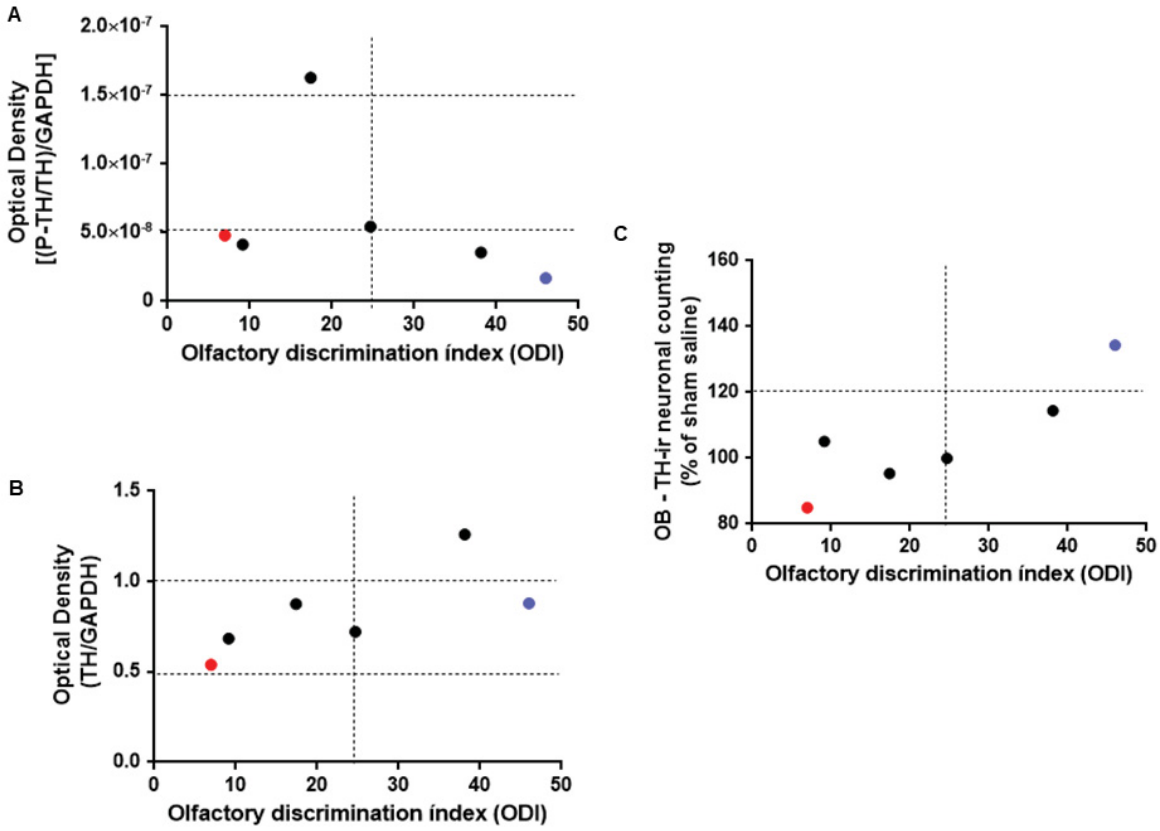

Figure 9. Male groups distribution diagrams**.** Mean groups are used to represent the groups distribution, based on X/Y factors. Red color = worse olfactory discrimination index group. Blue color = olfactory discrimination index higher performance group. Black color = average performance groups.

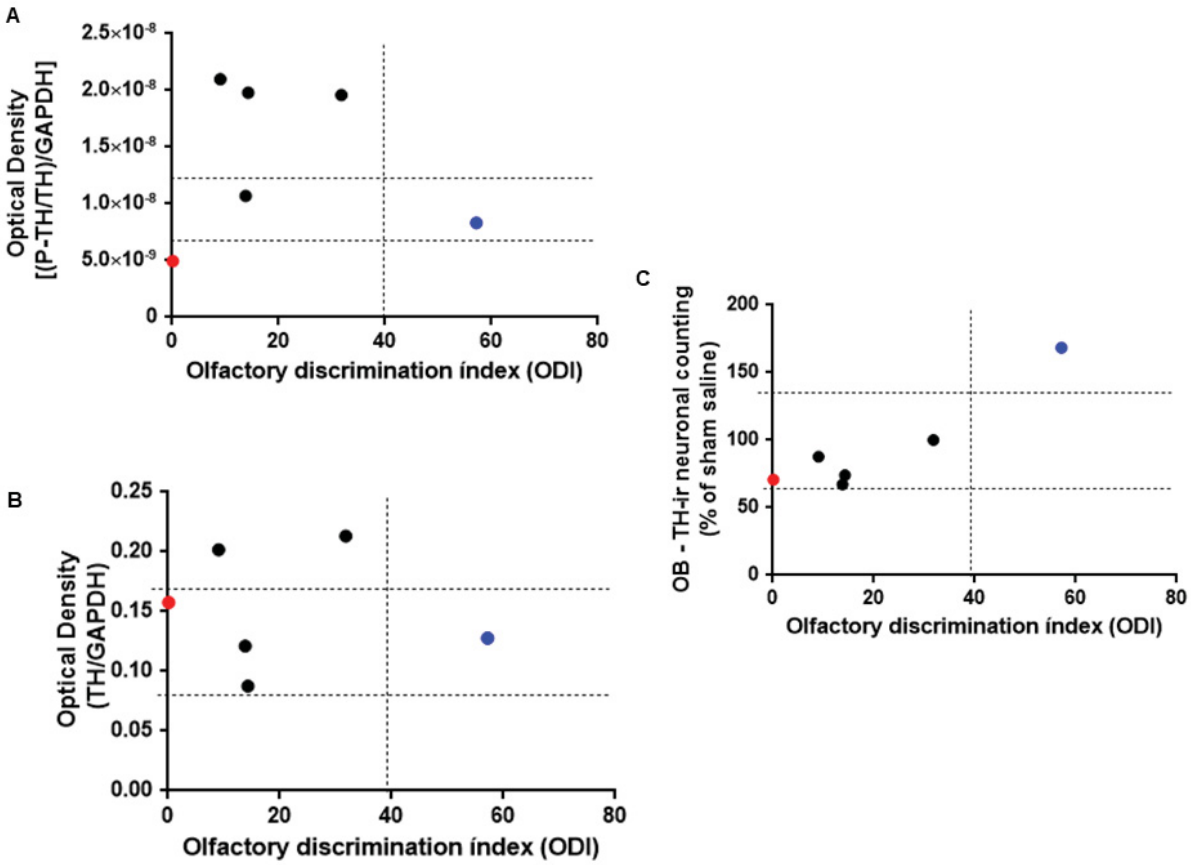

Figure 10. Female groups distribution diagrams**.** Mean groups are used to represent the groups distribution, based on X/Y factors. Red color = worse olfactory discrimination index group. Blue color = olfactory discrimination index higher performance group. Black color = average performance groups.

We observed that the A2a agonist slightly increased the TH phosphorylation in sham male and rotenone male and female groups. Besides, observing the groups distribution graphs, the higher ODI scores (blue dots) presented decreased p-TH expressions, for both males (Figure 9A) and females (Figure 10A). In addition, the lowest ODI scores (red dots) presented similar low p-TH densities, indicating that the TH phosphorylation was not sufficiently recruted by the drugs poorly impacting odor discrimination. Of note, chronic SCH58261 administration increases p-TH density in

the striatum (Hsu et al., 2010), particularly targeting the serine 31 residue of TH enzyme. In fact it has been reported that TH activity can be increased with phosphorylations at Ser8, Ser19, Ser31 and Ser40 residues *in vitro*, *in situ* and *in vivo*, although the main site is Ser40 (Dunkley, Bobrovskaya, Graham, Nagy-Felsobuki, & Dickson, 2004). Together, the OB immunohistochemistry and western blot analyzes suggest that the increased OB TH-ir neurons not represent mature cells, evidenced by low levels of OB ph-Ser 40-TH, which indicate low neuronal activity, impacting in low dopamine release. However, it is also discussed that low dopamine release, from periglomerular neurons, positively affects the olfactory discrimination (Rodrigues et al., 2014). In addition, we observed an increased TH phosphorylation only in male SCH 58261 sham group, compared to female SCH 58261 sham group, inversely related to the ODI, in which females presented higher ODI scores than males for the same treatment.

In conclusion, the current results suggest that A2a antagonist SCH58261 is capable to promote an improvement in the olfactory discrimination function in the rotenone animal model of PD, particularly in male rats, but not in females. Such result may be associated to the increased number of TH neurons within the OB, however, without a clear relation to increases in TH phosphorylation. It is possible to suggest that adenosinergic system is intimately involved in the olfactory process, possibly interacting with dopaminergic system, but further studies are necessary to understand the respective mechanisms involved and also the sex-related differences.

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Conflicts of Interest

There are no conflicts of interest.

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5 DISCUSSÃO GERAL

Diante dos resultados apresentados nos artigos, verificamos que em relação ao teste do campo aberto, o tratamento com cafeína promoveu um comportamento de hiperlocomoção em machos sham, similar ao que ocorre em outros estudos que utilizam a mesma dose (HSU; WANG; CHIU, 2010; MARIN et al., 2011). Ainda, vimos que a neurotoxina rotenona não afetou a locomoção de ratos machos, mas afetou em fêmeas, resultado que não parece ser exatamente um prejuízo motor, mas sim uma atividade basal aumentada em fêmeas do grupo sham salina, em comparação com o respectivo grupo em machos. Em relação ao tratamento com cafeína + nicotina, os ratos machos com lesão apresentaram uma hiperlocomoção, em comparação com todos os grupos rotenona e também com o grupo sham cafeína + nicotina, semelhante ao resultado encontrado por Garção e colaboradores (2013), que observaram uma sensibilização locomotora à nicotina (0,5 mg / kg) após uma injeção de antagonista do receptor A2a (ZM241385) em ratos (GARÇÃO et al., 2013).

Em relação à função olfatória, observamos um prejuízo ocasionado pela neurotoxina rotenona em machos, semelhante a dados prévios com o mesmo modelo (RODRIGUES et al., 2014). O oposto foi observado em fêmeas, que não apresentaram prejuízo algum na discriminação olfatória, corroborando dados epidemiológicos clínicos, nos quais homens apresentam prejuízos olfatórios decorrentes da DP muito mais pronunciados que mulheres (LIU et al., 2015). Ao contrário do que indicam os estudos epidemiológicos, o tratamento com nicotina não impediu que o prejuízo olfatório ocorresse, nem sozinho e nem em associação com a cafeína. O mecanismo que pode explicar esse resultado está ligado ao aumento da fosforilação da TH, o que de fato ocorreu, demonstrado nas analises de Western blot. Consequente ao aumento da fosforilação de TH no OB haveria o aumento da atividade de neurônios periglomerulares dopaminérgicos e maior ativação de receptores D2 inibitórios nos neurônios receptores de odor, que contribuem para a inibição do processamento olfatório (O'CONNOR; JACOB, 2008). O tratamento com cafeína, semelhante ao efeito causado pela microinfusão do antagonista A2a (SCH 58261) no BO, provavelmente causou leve aumento de fosforilação, suficiente para causar a melhora olfatória, mas não tão pronunciada a ponto de ativar efeitos dopaminérgicos inibitórios, como a nicotina e também o agonista A2a, que apesar de

não terem apresentado significância estatística, tiveram aumento de fosfo-TH mais pronunciado.

Apesar do tratamento com nicotina não ter sido promissor em relação ao distúrbio olfatório na dose utilizada, foi capaz de promover um efeito neuro-restaurador contra a lesão causada por rotenona na SNpc, tanto em machos quanto em fêmeas, semelhante a efeitos neuroprotetores observados em outros estudos (TAKEUCHI et al., 2009; MOUHAPE et al., 2018). A cafeína restaurou parcialmente a densidade neuronal na SNpc em ratos machos, semelhante ao resultado com administração de cafeína por 12 dias em ratos (SOLIMAN; FATHALLA; MOUSTAFA, 2016). Ainda, a quantificação de neurônios TH na CG do BO não foi afetada pela lesão intranigral em machos e também em fêmeas.

Com foco no distúrbio olfatório e no entendimento da função desempenhada pelo BO, planejamos os experimentos que deram origem ao artigo 2. Buscamos investigar, de forma seletiva, aguda e local, o envolvimento do sistema adenosinérgico na função olfatória.

Semelhante à cafeína, observamos melhora olfatória com a microinfusão do antagonista seletivo A2a SCH 58261 no BO, em machos, mas não em fêmeas, já que estas não apresentaram prejuízo olfatório. Os efeitos benéficos do antagonista A2a e da cafeína sobre a discriminação olfatória parecem estar relacionados, portanto, com o bloqueio desses receptores no BO. Ainda, o sistema adenosinérgico parece agir modulando o sistema dopaminérgico, por mecanismos não definidos, uma vez que o SCH 58261 levou ao aumento de expressão de neurônios TH na CG do BO, em machos rotenona e em fêmeas sham, os grupos com maiores médias no índice de discriminação olfatória. Apesar do aumento de expressão de neurônios TH na CG do BO observada em imunohistoquímica, não observamos aumento da expressão de TH na estrutura inteira e tampouco da fosforilação dessa enzima em análises de Western blot. A fosforilação de TH promovida por essas drogas parece ter tido pouco impacto na discriminação de odores, talvez pela administração aguda. Diferentemente, uma administração crônica de SCH58261 aumenta a densidade de fosfo-TH no estriado (HSU; WANG; CHIU, 2010).

As análises dos resultados referentes ao segundo artigo sugerem que o aumento dos neurônios TH-ir após infusão de SCH 58261 não representam células maduras, evidenciado por baixos níveis de TH fosforilada, indicando baixa atividade dopaminérgica. No entanto, se a liberação de DA for baixa, porém dentro de um

nível “ótimo”, resulta-se num efeito positivo sobre a discriminação olfatória, como observado na TDO.

6 CONSIDERAÇÕES FINAIS

Em relação à função locomotora, a cafeína causou hiperlocomoção e uma sensibilização locomotora à nicotina no tratamento duplo, para machos. As fêmeas apresentaram um prejuízo locomotor causado pela lesão intranigral em relação ao controle, cuja atividade basal já se mostrava mais alta do que em machos.

A memória de reconhecimento social não parece ter sido afetada com a rotenona, em machos. A administração de nicotina parece ter gerado algum grau de comprometimento cognitivo olfatório, por outro lado. Apesar de a rotenona ter provocado um prejuízo apresentado no teste de reconhecimento social para fêmeas do grupo salina, parece ser devido apenas à análise estatística utilizada; não representando de fato um prejuízo biológico.

A administração prolongada de cafeína e nicotina produziram efeitos diferentes no modelo animal utilizado, melhorando principalmente a função olfatória em machos (cafeína) ou restaurando a área degenerada na SNpc (nicotina) para machos e fêmeas. Ainda em relação ao prejuízo olfatório, o antagonista A2a apresentou efeito semelhante ao produzido pela cafeína, como esperado. O agonista A2a, por sua vez, prejudicou a função olfatória em machos e fêmeas. O modelo animal de rotenona intranigral, isoladamente, não gerou déficit olfatório significativo em fêmeas, semelhante ao que ocorre na clínica.

A degeneração de neurônios dopaminérgicos gerada pela rotenona na SNpc foi restaurada parcialmente pela cafeína e completamente com a nicotina e tratamento duplo, em machos; sendo restaurada apenas pela nicotina, em fêmeas. Dessa forma, sugerem-se novas evidências neuroprotetoras no modelo animal de DP induzido por rotenona intranigral.

A quantificação de neurônios TH na CG do BO não foi impactada pelo tratamento prolongado, exceto pela nicotina em machos sham. Contudo, em relação à administração aguda de drogas no BO, observamos um aumento desses neurônios para os grupos que receberam o antagonista A2a (machos rotenona e fêmeas sham), impactando diretamente na melhor discriminação olfatória observada pelo índice. Ainda, esse aumento parece representar células imaturas, sem aumento da expressão de TH e de fosforilação.

Os tratamentos prolongado com nicotina e cafeína + nicotina aumentaram a fosforilação da TH no BO, porém, de maneira exacerbada, prejudicando a função

olfatória em machos, sem apresentar prejuízo significativo em fêmeas. A administração aguda não produziu efeitos significativos quanto à fosforilação de TH no BO, em machos e fêmeas.

Concluindo, os resultados sugerem que existem diferenças entre os sexos no modelo animal utilizado, que ocorre de forma semelhante em uma população humana, o que significa que são necessárias terapias diferenciadas por sexo. Ainda, podemos inferir, indiretamente, que o sistema adenosinérgico está intimamente envolvido no processamento olfatório, possivelmente interagindo com o sistema dopaminérgico, porém são necessários novos estudos para melhor compreender os respectivos mecanismos envolvidos e também as diferenças relacionadas ao sexo.

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ANEXO 1 – PROOF DO ARTIGO "OLFACTION IN FEMALE WISTAR RATS IS INFLUENCED BY DOPAMINERGIC PERIGLOMERULAR NEURONS AFTER NIGRAL AND BULBAR LESIONS"

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Research report 1

Olfaction in female Wistar rats is influenced by dopaminergic periglomerular neurons after nigral and bulbar lesions

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Hyposmia is found in Parkinsonian patients decades before the onset of motor disorders. The same occurs with sleep disorders, especially infuencing rapid eye movement (REM) sleep, which affect a large percentage of people who have Parkinson's disease. These two disturbances presumably are closely related to a dopaminergic dysfunction. Thus, we propose that selective lesions, induced by rotenone, of the periglomerular neurons within the olfactory bulb or of the nigrostriatal pathway could result in hyposmia. In addition, we hypothesized that REM sleep deprivation (REMSD) could have potential to generate a synergistic olfactory impairment in both lesion paradigms. The results indicated that rotenone-induced nigrostriatal lesions in female Wistar rats were associated with odor preference changes, similar to hedonic tone impairment, but without a supposed potentiation triggered by REMSD. The nigrostriatal injury negatively affected olfaction performance, which was counteracted, functionally, by REMSD. However, injury to periglomerular neurons was less influenced by REMSD, as olfactory performance was restored after rebound sleep. We conclude that female rats present a pattern of olfactory discrimination/preference that is dependent on the activities of the nigrostriatal and the main olfactory pathways. *Behavioural Pharmacology* **00:000–000**

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Keywords: odor preference, olfactory bulb, Parkinson's disease, rat, rapid eye movement sleep deprivation, rotenone

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Introduction

Patients with Parkinson's disease (PD) often exhibit hyposmia and/or anosmia as an early phase sign that may be associated, according to studies in animal models, to disruptions in the main olfactory pathway (Tissingh *et al.*, 2001; Prediger *et al.*, 2006; Ansari and Johnson, 1975; Aurich *et al.*, 2017). Olfactory impairments are not usually considered as disabling as motor disturbances, but they are present in 90% of PD cases (Doty 2012; Doty *et al.* 1988). Remarkably, the topographical patterns of the first lesions develop at the olfactory bulb (OB) together with related portions of the anterior olfactory nucleus (Del Tredici *et al.*, 2002; Braak *et al.*, 2004). In addition to olfactory deficits, PD is also related to rapid eye movement (REM) sleep disorder, which is manifested in 25–50% of cases, significantly affecting the quality of life of these patients (Lima *et al.*, 2012; Lima, 2013). In fact, 24 h of sleep deprivation impairs olfactory performance in a smell identification task in humans (Killgore and McBride, 2006). Moreover, other studies have found similar olfactory impairment in patients with idiopathic REM sleep behavior disorder, suggesting a possible relation between this sleep phase and olfaction (Fantini *et al.*, 2006; Miyamoto *et al.*, 2009).

Despite the growing evidence of a dopaminergic influence in olfactory (Rodrigues *et al.*, 2014) and REM sleep mechanisms (Targa *et al.*, 2016, 2018), previously suggested by our group, and the recently described nigro-olfactory projection (Hoglinger *et al.*, 2015), several points still remain to be clarified. In this regard, tyrosine hydroxylase-immunoreactive (TH-ir) neurons, within the glomerular layer of the OB, appear to be the key players in odor discrimination, potentially contributing to the hyposmia/anosmia described in PD.

Hence, we propose that a selective lesion of these OB dopaminergic neurons could be associated with an early stage of the disease, thus producing hyposmia. We also hypothesize that the main olfactory pathway, which is potentially dependent on dopaminergic activity, might be drastically affected by a nigrostriatal lesion. In addition, REM sleep deprivation (REMSD) could also trigger, by itself (owing to dopaminergic D2 supersensitivity) (Tufik *et al.*, 1978; Tufik 1981a), some level of hyposmia, with a potential to generate a synergistic olfactory impairment in both lesion paradigms.

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